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Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as a potential biocontrol bacteria isolated from Baluran National Park, East Java, Indonesia

Author(s) name:

Salamun, Rizky Danang Susetyo, Ni'matuzahroh, Fatimah, Almando Geraldi, Agus Supriyanto, Tri Nurhariyati, Farah Aisyah Nafidiastri, Nabilatun Nisa', Endarto

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Novelty:

The BK7.1 isolate was obtained from the natural soil of Baluran National Park, one of the natural conservation areas in Indonesia. Potency test, proven larvicidal effect on *Aedes aegypti* larvae. Researchers are interested in further research and the results of molecular identification show that BK7.1 is *B. subtilis*, but it does not produce Cry toxin. From the literature study, biosurfactants contain surfactin, iturin, and fengisin compounds which can be developed as biocontrol agents for antibacterial, antifungal, and larvicidal effects. The role of biosurfactants has not been widely discussed in biological control and the genes involved in biosurfactant biosynthesis can be developed for cloning of coding genes that can be used by large-scale industries, so that BK7.1 isolate is prospective as a biocontrol agent in plant pathogens and plant pests.

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Salamun

Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as a potential biocontrol bacteria isolated from Baluran National Park, East Java, Indonesia

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Manuscript received: 27 01 2023. Revision accepted: ..... 2016.

Abstract. Using biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research was focused on identifying the species and genetic relationship of entomopathogenic *Bacillus* sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Screening of biosurfactants by tested hemolytic activity, surface tension and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing the production of biosurfactants in various substrates were conducted. The molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method of *Bacillus* sp. BK7.1 has a genetic similarity of 98.68% with *B. subtilis* subsp. inaquosorum strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension and increased emulsification activities, and produced biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that *Bacillus* sp. BK7.1 had srfAA and srfAD genes encoding surfactin biosynthesis, the potential bacteria to produce bioinsecticide compounds. Thus, the indigenous entomopathogenic *B. subtilis* BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

Keywords: Biosurfactant production, crop protection, entomopathogenic *Bacillus subtilis* BK7.1, hemolytic activity, srfAA-srfAD gene.

Running Title: Biosurfactant production of *Bacillus subtilis* BK7.1

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is broadly used (Korrat et al. 2012, Safni et al. 2018). However, chemical insecticides have a negative impact on disease vector control and pest control because it causes insect resistance (Silva et al. 2018). Biocontrol methods are obtainable to resolve these problems. Entomopathogens from microorganisms act as natural enemies that can produce toxic metabolites towards insect pests and plants pathogen. Biocontrol methods can be used as an alternative to fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. This method does not cause pollution and is environmentally friendly (Thomas 2017).

Biocontrol agents using *Bacillus* strains are methods that have been widely developed because they are proven to be environmentally friendly (Bergamasco et al. 2013, Syaharuddin et al. 2018). Group of bacteria, fungi and yeasts have produced biosurfactants (Santos et al. 2018). Biosurfactants can be synthesized by several groups of microbes and can act

as a substitute for non-biodegradable and non-environmentally friendly synthetic surfactants (Moro et al. 2018). The biosurfactant produced by *Bacillus* is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features, such as high levels of biodegradability and optimal activity under extreme conditions (Banat et al. 2010, Kedher et al. 2017). Following previous study, *B. subtilis*, *B. amyloliquefaciens*, and *B, velezensis* produces biosurfactant and efficient to be biocontrol agent against different targets (Geetha et al. 2010, Ghribi et al. 2012, Revathi et al. 2013, Nafidiastri et al. 2021). *Bacillus* sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (Mongkolthanaruk 2012). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and trigger systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016, Pele et al. 2019, Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016, Chaves et al. 2018, Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic *Bacillus* are still being carried out to find the safest way to control disease vectors caused by mosquitoes. The results of screening tests for potential initial toxicity against *A. aegypti* larvae have reported that 68 entomopathogenic *Bacillus* sp. has been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). *Bacillus* sp. BK5.2, a molecular identification, has been carried out, as *B. thuringiensis* BK5.2 which produces entomopathogenic cry toxin (Salamun et al. 2021), during the identification of *Bacillus* sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of *Bacillus* sp. BK7.1 as an entomopathogenic bacteria. In this study, genetic characteristics were carried out to determine the species and the relationship of species in the phylogenetic tree, detection of biosurfactant coding genes, screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on several substrates.

MATERIALS AND METHODS

Identification 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r), examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and visualized under ultraviolet light, then purified and sequenced. Amplicon result was then aligned and contigs were developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with Du Nouy Tensiometer, with 50% Tween 20 as a positive control and Nutrient Broth as a negative control. The decrease in the surface tension value of 10 mN/m indicated the potential to produce biosurfactants. The emulsification activity was measured by inserting a 2 mL supernatant fraction and kerosene in a test tube. This mixture was stirred on Vortex Mixer

for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection srfAA and srfAD surfactin gene

Amplification of the srfAA and srfAD surfactin genes of *Bacillus* sp. BK7.1 using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3' and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for srfAA gene (Chung et al. 2008). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATTGGATGCT 3' for srfAD gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolved one by one, 3 g (NH4)2SO4, 10 g NaCl, 0.2 g MgSO4.7H2O, 0.01 g CaCl2, 0.001 g MnSO4.H2O, 0.001 g H3BO3, 0.001 g ZnSO4.7H2O, 0.001 g CuSO4.5H2O, 0.005 g CoCl2.6H2O, and 0.001 g NaMoO4.2H2O into 900 mL distilled water, respectively. The elements phosphate and iron are made separately. The phosphate elements dissolved 5 g of KH2PO4 and 2 g of K2HPO4 into 50 mL of distilled water, while the iron element dissolved 0.0006 g of FeSO4.7H2O into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hours with an agitation of 130 rpm. Every 24 hours, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hours incubation.

RESULTS AND DISCUSSION

Results

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Fig. 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, homology level of 98.68% (Table 1).

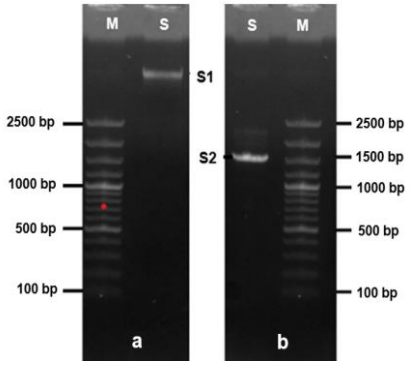

Fig. 1 The electrophoresis results of DNA genome (**a**) and 16S rRNA gene (**b**) of *Bacillus* sp. BK7.1 on 1% agarose gel. Descriptions: **M** 100 bp DNA marker, **S** sample, **S1** sample of DNA genome, **S2** sample of 16SrRNA gene

Table 1 The species of *Bacillus* sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

No.	Species	Accession No.	E value	%ID	Query Cover (%)
1	*Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28	NR_104873.1	0.0	98.68	99
2	*Bacillus subtilis* strain DSM 10	NR_027552.1	0.0	98.61	99
3	*Bacillus subtilis* strain JCM 1465	NR_113265.1	0.0	98.61	99

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Fig. 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

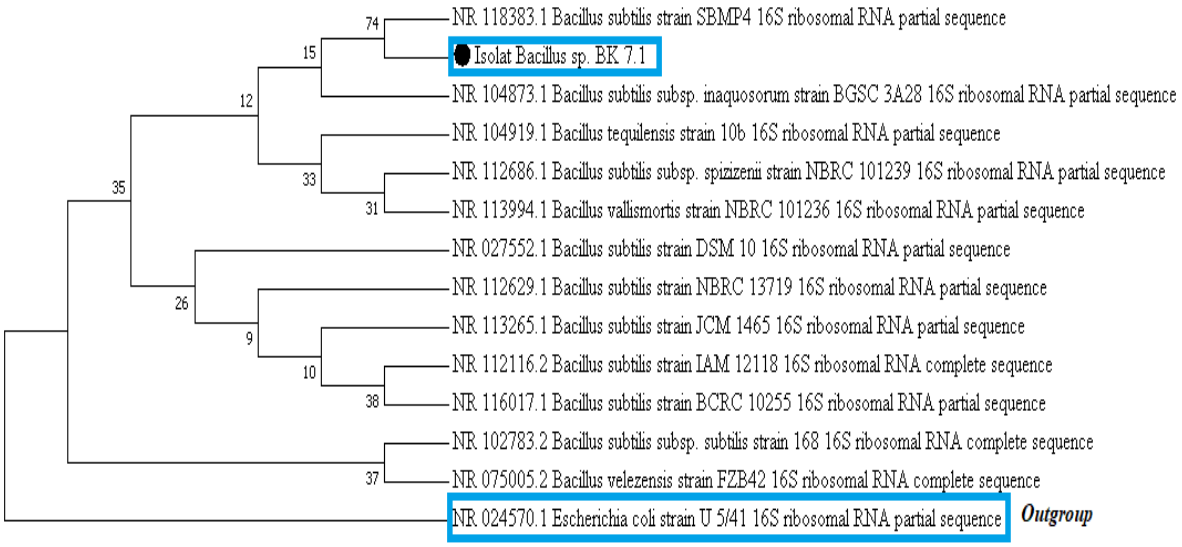

Fig. 2 Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83 mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which is left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it has decreased by 21.92% (Fig. 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

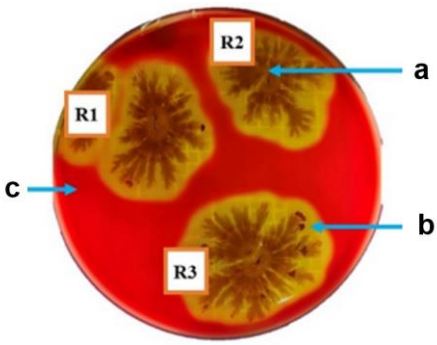

Fig. 3 Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. Descriptions: **a** isolate, **b** clear zone around the colony, **c** blood agar plate, **R** Replicates.

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

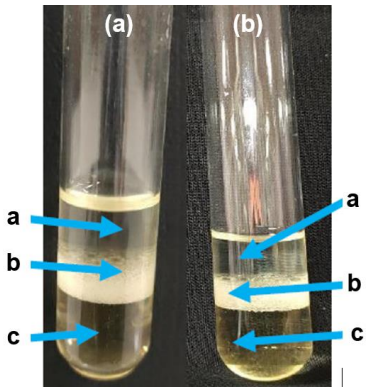

Fig. 4 The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. Descriptions: **a** kerosene, **b** emulsion, **c** isolate, (**a**) 1 hour of exposure, (**b**) 24 hours of exposure.

Detection srfAA and srfAD surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as srfAA gene, and 723 bp expected as srfAD gene (Fig. 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

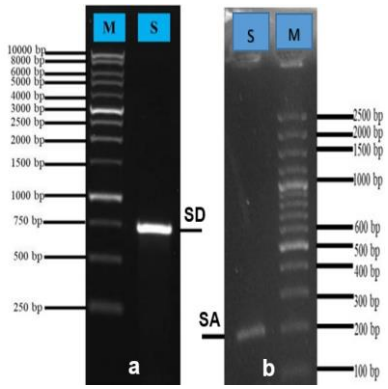

Fig. 5 The electrophoresis results of srfAD (**a**) and srfAA (**b**) surfactin gene amplification of *Bacillus subtilis* BK7.1. Description: **M** 100 bp DNA marker, **SA** sample of srfAA surfactin gene 201 bp, **SD** sample of srfAD surfactin gene 729 bp

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of srfAA and srfAD protein isolates of *Bacillus subtilis* BK 7.1

No.	Protein	Species	Accession No.	E value	%ID	Query Cover (%)

1.	surfactin non-ribosomal peptide synthetase srfAA	*Bacillus subtilis* inaquosorum	WP_060397903.1	9e-34	91.04	100
2.	surfactin biosynthesis thioesterase SrfAD	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Fig. 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Fig. 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 h incubation, isolates still showed an exponential phase, and 96 h incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 h incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hours of incubation (Fig. 6bc), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hours of incubation. Decreased in surface tension values are shown in Fig. 6d.

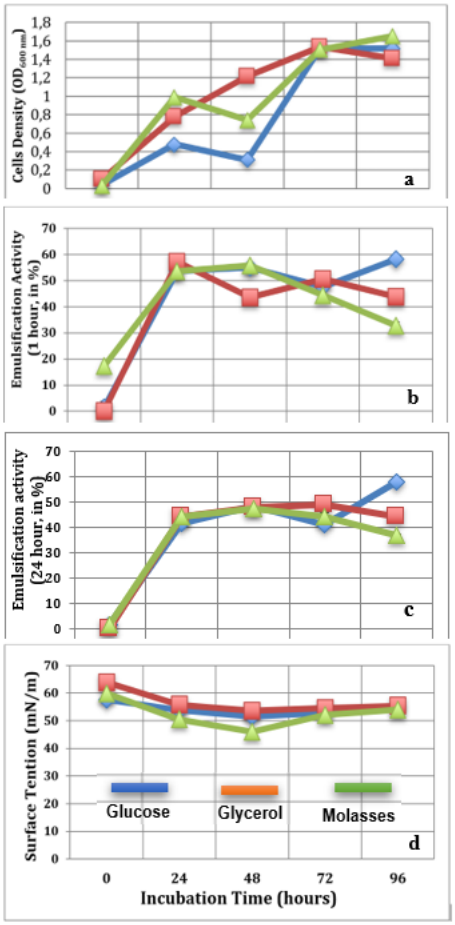

Fig. 6 Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: **a** cells density, **b** emulsification activity (1 hour), **c** emulsification activity (24 hours), **d** surface tension value

Discussions

The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Fig. 1). Based on molecular identification, *Bacillus* sp. BK7.1, which similarity to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, homology level of 98.68%. The gene of 16S rRNA can be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing is a fast and

accurate method for bacterial identification. Bacteria represent the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019, Srinivasan et al. 2015). The similarity is less than 100% because there are variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research conducted that *B. subtilis* strain SBMP4 can control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted and grown in extreme environmental conditions, forms endospores are resistant to stress, and has secreted various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic is the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016, Mishra and Arora 2018). Surfactin produced by *B. subtilis* is one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013, Gudina et al. 2016).

Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain can kill adult mosquitoes (Geetha et al. 2012). The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity occurs through two different mechanisms, at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes it will causes osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria can produce biosurfactants if they can reduce surface tension values by ≥ 10 mN/m (Francy et al. 1991). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* is caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 causes the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin can be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (Geetha et al. 2010).

The emulsification index value of *B. subtilis* BK7.1 is a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure causes surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin are involved in cell attachment and cause membrane disruption (Raaijmakers et al. 2010). The ability of surfactin to bind Ca^{2+} causes a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Kedher et al. 2015, Kedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation tends to decrease compared to 24 hours observation. This difference has shown that the emulsion is unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, are efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, are more effective in stabilizing oil-in-water emulsions as emulsifiers (Calvo et al. 2009). This result is supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affect the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species have srfAA gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (Jacques 2011, Plaza et al. 2015). The nonribosomal peptide synthetase complex is coded by srfAA and srfAD gene known as surfactin synthetase. The srfAA and srfAD genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase is an activating enzyme for the srfA multienzyme complex. The srfAA, srfAB, srfAC, and srfAD genes are involved in the assembly of heptamodular non-ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain

and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene transforms surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by srfAA and srfAD gene (Jacques 2011, Plaza et al. 2015). The Table 3 showed that the similarity results have a value of 91.04%, because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity can cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. Marine bacteria on mineral salt media containing different carbon sources, higher emulsification activity of glycerol and starch substrates than glucose and sucrose substrates, with a value range of (E24) 45-85% (Das et al. 2008). Different reports showed that the emulsification activity of *B. subtilis* 573 to 48.4% (Pereira et al. 2013), also the emulsification activity of *B. subtilis* up to 38.3% (Zhu et al. 2016). The production of biosurfactant by *B. circulans* in glycerol and anthracene-supplemented glycerol substrate has been able to emulsify various hydrocarbons, such as diesel oil, hexadecane, kerosene, benzene, and gasoline in the range of 30-80% (Das et al. 2008).

Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al. 2014). The difference in surface tension reduction is caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide sequences between bacteria species affect the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate is influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. inaquosorum strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The srfAA and srfAD genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (Penelitian Unggulan Fakultas) 2022-2023. The authors grate full to the Dean of the Faculty of Science and Technology (Fakultas Sains dan Teknologi) and Head of Research and Community Services Institutions (Lembaga Penelitian dan Pengabdian Masyarakat), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as a potential biocontrol bacteria isolated from Baluran National Park, East Java, Indonesia

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Novelty:

The BK7.1 isolate was obtained from the natural soil of Baluran National Park, one of the natural conservation areas in Indonesia. Potency test, proven larvicidal effect on Aedes aegypti larvae. Researchers are interested in further research and the results of molecular identification show that BK7.1 is B. subtilis, but it does not produce Cry toxin. From the literature study, biosurfactants contain surfactin, iturin, and fengisin compounds which can be developed as biocontrol agents for antibacterial, antifungal, and larvicidal effects. The role of biosurfactants has not been widely discussed in biological control and the genes involved in biosurfactant biosynthesis can be developed for cloning of coding genes that can be used by large-scale industries, so that BK7.1 isolate is prospective as a biocontrol agent in plant pathogens and plant pests.

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Salamun

Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as a potential biocontrol bacteria isolated from Baluran National Park, East Java, Indonesia

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Manuscript received: 27 01 2023. Revision accepted: ..... 2016.

Abstract. Using biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research aimed to identify ~~focused on identifying~~ the species and genetic relationship, hemolytic activity, detection of coding genes, and trial production of biosurfactants on various substrates of entomopathogenic *Bacillus* sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Screening of biosurfactants by ~~tested~~ testing hemolytic activity, surface tension and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing the production of biosurfactants in various substrates were conducted. The results of the molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method of *Bacillus* sp. BK7.1 has a genetic similarity of 98.68% with *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension, and increased emulsification activities, and produced biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that *Bacillus* sp. BK7.1 had srfAA and srfAD genes encoding surfactin biosynthesis, the potential bacteria to produce bioinsecticide compounds. Based on these studies ~~Thus,~~ the indigenous entomopathogenic *B. subtilis* BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

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Keywords: Biosurfactant production, crop protection, entomopathogenic *Bacillus subtilis* BK7.1, hemolytic activity, srfAA-srfAD gene.

Running Title: Biosurfactant production of *Bacillus subtilis* BK7.1

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is broadly used (~~Korrat et al. 2012~~, Safni et al. 2018). However, chemical insecticides have a negative impact on disease vector control and pest control because it causes insect resistance (~~Silva et al. 2018,~~ Şengül et al. 2022). Biocontrol methods are obtainable to resolve these problems. Entomopathogens from microorganisms act as natural enemies that can produce toxic metabolites towards insect pests and plants pathogen. Biocontrol methods can be used as an alternative to fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. This method does not cause pollution and is environmentally friendly (Thomas 2017).

Biocontrol agents using *Bacillus* strains are methods that have been widely developed because they are proven to be environmentally friendly (Bergamasco et al. 2013, Syaharuddin et al. 2018). Group of bacteria, fungi and yeasts have

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produced biosurfactants (Santos et al. 2018). Biosurfactants can be synthesized by several groups of microbes and can act as a substitute for non-biodegradable and non-environmentally friendly synthetic surfactants (Moro et al. 2018). The biosurfactant produced by *Bacillus* is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features, such as high levels of biodegradability and optimal activity under extreme conditions (~~Banat et al. 2010,~~ Khedher et al. 2017). Following previous study, *B. subtilis*, *B. amyloliquefaciens*, and *B, velezensis* produces biosurfactant and efficient to be biocontrol agent against different targets (~~Geetha et al. 2010, Ghribi et al. 2012,~~ Revathi et al. 2013, Nafidiastri et al. 2021). *Bacillus* sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (~~Mongkolthanaruk 2012,~~ Théatre et al. 2021). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and trigger systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016, Pele et al. 2019, Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016, Chaves et al. 2018, Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic *Bacillus* are still being carried out to find the safest way to control disease vectors caused by mosquitoes. The results of screening tests for potential initial toxicity against *A. aegypti* larvae have reported that 68 entomopathogenic *Bacillus* sp. has been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). *Bacillus* sp. BK5.2, a molecular identification, has been carried out, as *B. thuringiensis* BK5.2 which produces entomopathogenic cry toxin (Salamun et al. 2021), during the identification of *Bacillus* sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of *Bacillus* sp. BK7.1 as an entomopathogenic bacteria. In this study, genetic characteristics were carried out to determine the species and the relationship of species in the phylogenetic tree, detection of biosurfactant coding genes, screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on several substrates.

MATERIALS AND METHODS

Identification 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r), examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and visualized under ultraviolet light, then purified and sequenced. Amplicon result was then aligned and contigs were developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with Du Nouy Tensiometer, with 50% Tween 20 as a positive control and Nutrient Broth as a negative control. The decrease in the surface tension value of 10 mN/m indicated the potential to produce biosurfactants. The emulsification activity was

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measured by inserting a 2 mL supernatant fraction and kerosene in a test tube. This mixture was stirred on Vortex Mixer for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection srfAA and srfAD surfactin gene

Amplification of the srfAA and srfAD surfactin genes of *Bacillus* sp. BK7.1 using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3' and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for srfAA gene (~~Chung et al. 2008,~~ Mora et al. 2020). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATTGGATGCT 3' for srfAD gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

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Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolved one by one, 3 g (NH4)2SO4, 10 g NaCl, 0.2 g MgSO4.7H2O, 0.01 g CaCl2, 0.001 g MnSO4.H2O, 0.001 g H3BO3, 0.001 g ZnSO4.7H2O, 0.001 g CuSO4.5H2O, 0.005 g CoCl2.6H2O, and 0.001 g NaMoO4.2H2O into 900 mL distilled water, respectively. The elements phosphate and iron are made separately. The phosphate elements dissolved 5 g of KH2PO4 and 2 g of K2HPO4 into 50 mL of distilled water, while the iron element dissolved 0.0006 g of FeSO4.7H2O into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hours with an agitation of 130 rpm. Every 24 hours, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hours incubation.

RESULTS AND DISCUSSION

Results

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Fig. 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, homology level of 98.68% (Table 1).

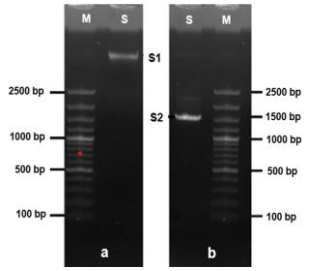


Fig. 1 The electrophoresis results of DNA genome (**a**) and 16S rRNA gene (**b**) of *Bacillus* sp. BK7.1 on 1% agarose gel. Descriptions: **M** 100 bp DNA marker, **S** sample, **S1** sample of DNA genome, **S2** sample of 16SrRNA gene

Table 1 The species of *Bacillus* sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

No.	Species	Accession No.	E value	%ID	Query Cover (%)
1	*Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28	NR_104873.1	0.0	98.68	99
2	*Bacillus subtilis* strain DSM 10	NR_027552.1	0.0	98.61	99
3	*Bacillus subtilis* strain JCM 1465	NR_113265.1	0.0	98.61	99

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Fig. 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

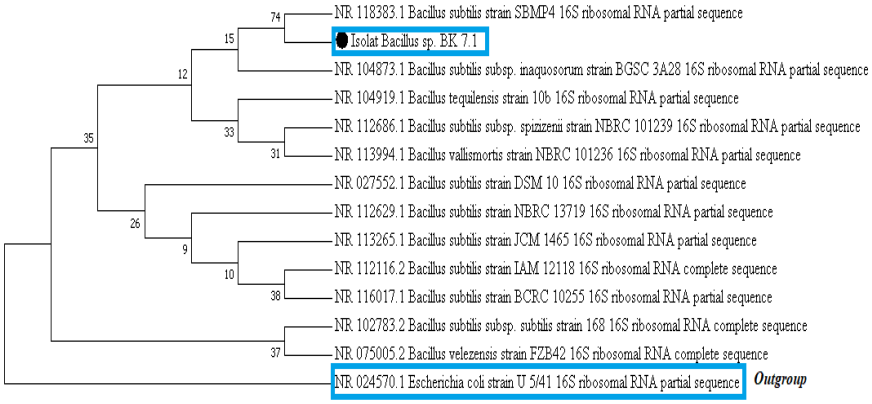


Fig. 2 Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83 mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which is left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it has decreased by 21.92% (Fig. 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

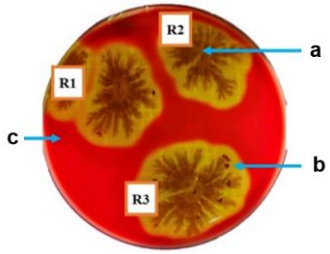


Fig. 3 Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. Descriptions: **a** isolate, **b** clear zone around the colony, **c** blood agar plate, **R** Replicates.

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

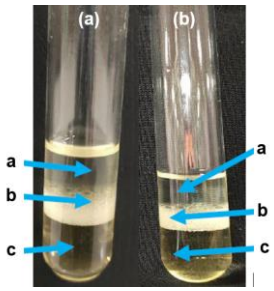


Fig. 4 The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. Descriptions: **a** kerosene, **b** emulsion, **c** isolate, (**a**) 1 hour of exposure, (**b**) 24 hours of exposure.

Detection srfAA and srfAD surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as srfAA gene, and 723 bp expected as srfAD gene (Fig. 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

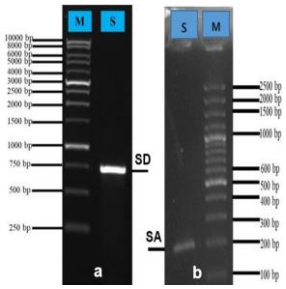


Fig. 5 The electrophoresis results of srfAD (**a**) and srfAA (**b**) surfactin gene amplification of *Bacillus subtilis* BK7.1. Description: **M** 100 bp DNA marker, **SA** sample of srfAA surfactin gene 201 bp, **SD** sample of srfAD surfactin gene 729 bp

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of srfAA and srfAD protein isolates of *Bacillus subtilis* BK 7.1

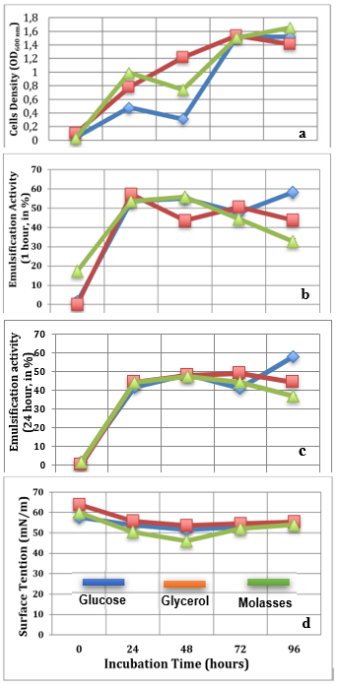
No.	Protein	Species	Accession No.	E value	%ID	Query Cover (%)

1.	surfactin non-ribosomal peptide synthetase srfAA	*Bacillus subtilis* inaquosorum	WP_060397903.1	9e-34	91.04	100
2.	surfactin biosynthesis thioesterase SrfAD	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Fig. 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Fig. 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 h incubation, isolates still showed an exponential phase, and 96 h incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 h incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hours of incubation (Fig. 6bc), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hours of incubation. Decreased in surface tension values are shown in Fig. 6d.



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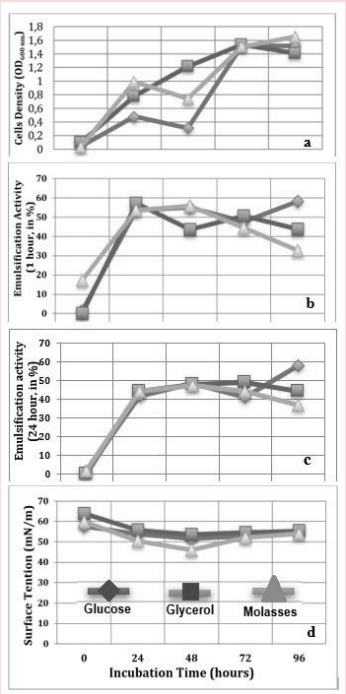

Fig. 6 Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: **a** cells density, **b** emulsification activity (1 hour), **c** emulsification activity (24 hours), **d** surface tension value

Discussions

The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Fig. 1). Based on molecular identification, *Bacillus* sp. BK7.1, which similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28, homology level of 98.68%. The gene of 16S rRNA can be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing is a fast and

accurate method for bacterial identification. Bacteria represent the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019, Srinivasan et al. 2015). The similarity is less than 100% because there are variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research conducted that *B. subtilis* strain SBMP4 can control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted and grown in extreme environmental conditions, forms endospores are resistant to stress, and has secreted various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic is the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016, Mishra and Arora 2018). Surfactin produced by *B. subtilis* is one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013, Gudina et al. 2016).

Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain can kill adult mosquitoes (~~Geetha et al. 2012~~). The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity occurs through two different mechanisms, at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes it will causes osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria can produce biosurfactants if they can reduce surface tension values by ≥ 10 mN/m (~~Francy et al. 1991,~~ de Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* is caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 causes the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin can be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (~~Geetha et al. 2010~~, Guimarães et al. 2019).

The emulsification index value of *B. subtilis* BK7.1 is a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure causes surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin are involved in cell attachment and cause membrane disruption (~~Raaijmakers et al. 2010,~~ Chen et al. 2022). The ability of surfactin to bind Ca^{2+} causes a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Khedher et al. 2015, Khedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation tends to decrease compared to 24 hours observation. This difference has shown that the emulsion is unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, are efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, are more effective in stabilizing oil-in-water emulsions as emulsifiers (~~Calvo et al. 2009,~~ Uzoigwe et al. 2015). This result is supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affect the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species have srfAA gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (~~Jacques 2011,~~ Plaza et al. 2015). The nonribosomal peptide synthetase complex is coded by srfAA and srfAD gene known as surfactin synthetase. The srfAA and srfAD genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase is an activating enzyme for the srfA multienzyme complex. The srfAA, srfAB, srfAC, and srfAD genes are involved in the assembly of heptamodular non-

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ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene transforms surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by srfAA and srfAD gene (~~Jacques 2011,~~ Plaza et al. 2015). The Table 3 showed that the similarity results have a value of 91.04%, because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity can cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. The higher emulsification activity from *B. subtilis* 573 to 48.4% (Pereira et al. 2013), *B. subtilis* 21332 up to 55.2% (Zhu et al. 2016), and *B. subtilis* N3-4P up to 38.3% (Zhu et al. 2016) on mineral salt media containing using different carbon sources than glycerol. ~~The production of biosurfactant by *B. circulans* in glycerol and anthracene-supplemented glycerol substrate has been able to emulsify various hydrocarbons, such as diesel oil, hexadecane, kerosene, benzene, and gasoline in the range of 30–80% (Das et al. 2008).~~ The production of biosurfactant by *Bacillus nealsonii* S2M in glycerol substrate has been able to emulsify various hydrocarbons in 55% (Phulpoto et al. 2020).

Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al. 2014). The difference in surface tension reduction is caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide sequences between bacteria species affect the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate is influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. inaquosorum strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The srfAA and srfAD genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (Penelitian Unggulan Fakultas) 2022-2023. The authors grate full to the Dean of the Faculty of Science and Technology (Fakultas Sains dan Teknologi) and Head of Research and Community Services Institutions (Lembaga Penelitian dan Pengabdian Masyarakat), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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Submissions

Workflow | Publication

Submission | Review | Copyediting | Production

Round 1 | Round 2 | Round 3 | Round 4 | Round 5

Round 3 Status

New reviews have been submitted and are being considered by the editor.

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Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as a potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia

Abstract. BUsing biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research aimed to identify the species and genetic relationship, hemolytic activity, detect detection of coding genes, and trial production of biosurfactants on various substrates of entomopathogenic Bacillus sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Biosurfactant screening was carried out by testing hemolytic activity, surface tension, and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing biosurfactant production in various substrates. Screening of biosurfactants by testing hemolytic activity, surface tension and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing the production of biosurfactants in various substrates were conducted. The results of the molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method of for Bacillus sp. BK7.1 has a genetic similarity of 98.68% with B. subtilis subsp. inaquosorum strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension, and increased emulsification activities, and the production ofproduced biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that Bacillus sp. BK7.1 had srfAA and srfAD genes encoding surfactin biosynthesis, giving it the potential bacteria to produce bioinsecticide compounds. Based on these studies, the indigenous entomopathogenic B. subtilis BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

Keywords: Biosurfactant production, crop protection, entomopathogenic *Bacillus subtilis* BK7.1, hemolytic activity, srfAA-srfAD gene.

Running Title: Biosurfactant production of Bacillus subtilis BK7.1

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is widely broadly used (Safni et al. 2018). However, chemical insecticides have a negative impact on disease vector control and pest control because they causeit causes insect resistance (Şengül et al. 2022). Biocontrol methods are available obtainable to resolve these problems. Entomopathogens are natural enemies that can produce toxic metabolites against insect pests and plant pathogens. Entomopathogens from microorganisms act as natural enemies that can produce toxic metabolites towards

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~~insect pests and plants pathogen.~~ Biocontrol methods can be used as an alternative to fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. This method does not cause pollution and is environmentally friendly (Thomas 2017).

Biocontrol agents using Bacillus strains are methods that have been widely developed because they are proven to be environmentally friendly (ttps://doi.org/10.3390/microorganisms8020232; https://www.nature.com/articles/s41598-021-93285-7, Bergamasco et al. 2013, Syaharuddin et al. 2018). A Ggroup of bacteria, fungi, and yeasts have produced biosurfactants (Santos et al. 2018). Several groups of microbes can synthesize biosurfactants, which can be used to replace non-biodegradable and environmentally unfriendly synthetic surfactants ~~Biosurfactants can be synthesized by several groups of microbes and can act as a substitute for non-biodegradable and non-environmentally friendly synthetic surfactants~~ (Moro et al. 2018). The biosurfactant produced by Bacillus is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features, such as high levels of biodegradability and optimal activity under extreme conditions (Khedher et al. 2017). Following previous studies~~y~~, B. subtilis, B. amyloliquefaciens, and B. velezensis produce~~s~~ biosurfactant and are efficient ~~to be~~ biocontrol agents against different targets (Revathi et al. 2013, Nafidiastri et al. 2021). Bacillus sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (Théatre et al. 2021). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and triggering systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016, Pele et al. 2019, Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016, Chaves et al. 2018, Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature conditions often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic Bacillus are still being carried out to find the safest way to control disease vectors caused by mosquitoes. The results of screening tests for potential initial toxicity against A. aegypti larvae have reported that 68 entomopathogenic Bacillus sp. ~~has~~ have been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). Bacillus sp. BK5.2, a molecular identification, has been carried out, as B. thuringiensis BK5.2 which produces an entomopathogenic cry toxin (Salamun et al. 2021). The~~, during the~~ identification of Bacillus sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of Bacillus sp. BK7.1 as an entomopathogenic bacteria. Genetic characteristics were used in this study to determine the species and their relationships in the phylogenetic tree, as well as the detection of biosurfactant coding genes and the screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on various substrates.~~In this study, genetic characteristics were carried out to determine the species and the relationship of species in the phylogenetic tree, detection of biosurfactant coding genes, screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on several substrates.~~

MATERIALS AND METHODS

Identification 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r), examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and

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visualized under ultraviolet light, then purified and sequenced. Amplicon result was then aligned and contigs were developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with Du Nouy Tensiometer, with 50% Tween 20 as a positive control and Nutrient Broth as a negative control. The decrease in the surface tension value of 10 mN/m indicated the potential to produce biosurfactants. The emulsification activity was measured by inserting a 2 mL supernatant fraction and kerosene in a test tube. This mixture was stirred on Vortex Mixer for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection srfAA and srfAD surfactin gene

Amplification of the srfAA and srfAD surfactin genes of *Bacillus* sp. BK7.1 using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3' and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for srfAA gene (Mora et al. 2020). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATTGGATGCT 3' for srfAD gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolved one by one, 3 g (NH4)2SO4, 10 g NaCl, 0.2 g MgSO4.7H2O, 0.01 g CaCl2, 0.001 g MnSO4.H2O, 0.001 g H3BO3, 0.001 g ZnSO4.7H2O, 0.001 g CuSO4.5H2O, 0.005 g CoCl2.6H2O, and 0.001 g NaMoO4.2H2O into 900 mL distilled water, respectively. The elements phosphate and iron are made separately. The phosphate elements dissolved 5 g of KH2PO4 and 2 g of K2HPO4 into 50 mL of distilled water, while the iron element dissolved 0.0006 g of FeSO4.7H2O into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hours with an agitation of 130 rpm. Every 24 hours, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hours incubation.

RESULTS AND DISCUSSION

Results

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Fig. 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, homology level of 98.68% (Table 1).

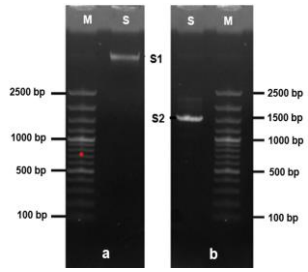

Fig. 1 The electrophoresis results of DNA genome (**a**) and 16S rRNA gene (**b**) of *Bacillus* sp. BK7.1 on 1% agarose gel. Descriptions: **M** 100 bp DNA marker, **S** sample, **S1** sample of DNA genome, **S2** sample of 16SrRNA gene

Table 1 The species of *Bacillus* sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

No.	Species	Accession No.	E value	%ID	Query Cover (%)
1	*Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28	NR_104873.1	0.0	98.68	99
2	*Bacillus subtilis* strain DSM 10	NR_027552.1	0.0	98.61	99
3	*Bacillus subtilis* strain JCM 1465	NR_113265.1	0.0	98.61	99

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Fig. 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

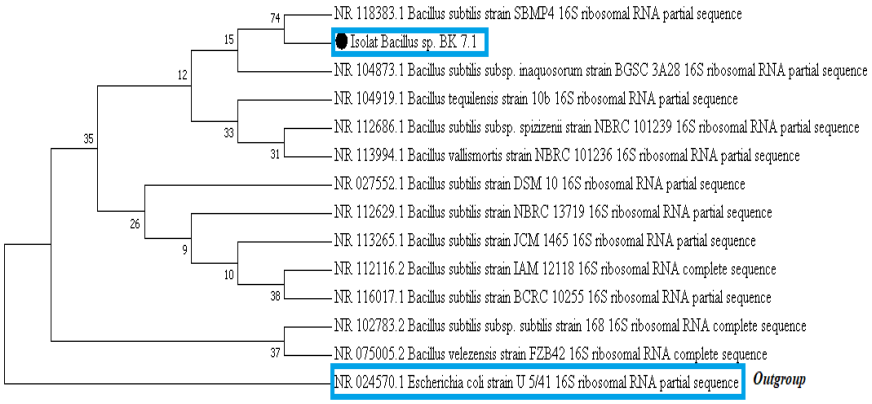

Fig. 2 Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83

mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which is left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it has decreased by 21.92% (Fig. 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

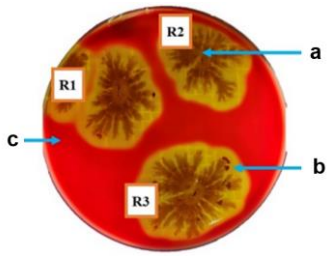

Fig. 3 Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. Descriptions: **a** isolate, **b** clear zone around the colony, **c** blood agar plate, **R** Replicates.

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

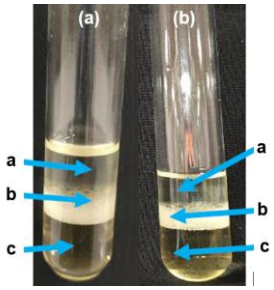

Fig. 4 The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. Descriptions: **a** kerosene, **b** emulsion, **c** isolate, (**a**) 1 hour of exposure, (**b**) 24 hours of exposure.

Detection srfAA and srfAD surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as srfAA gene, and 723 bp expected as srfAD gene (Fig. 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

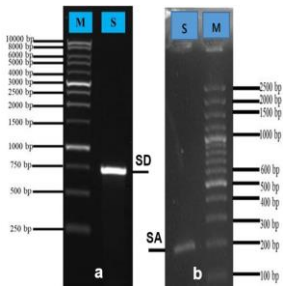

Fig. 5 The electrophoresis results of srfAD (**a**) and srfAA (**b**) surfactin gene amplification of *Bacillus subtilis* BK7.1. Description: **M** 100 bp DNA marker, **SA** sample of srfAA surfactin gene 201 bp, **SD** sample of srfAD surfactin gene 729 bp

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of srfAA and srfAD protein isolates of *Bacillus subtilis* BK 7.1

No.	Protein	Species	Accession No.	E value	%ID	Query Cover (%)
1.	surfactin non-ribosomal peptide synthetase srfAA	*Bacillus subtilis* inaquosorum	WP_060397903.1	9e-34	91.04	100
2.	surfactin biosynthesis thioesterase SrfAD	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Fig. 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Fig. 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 h incubation, isolates still showed an exponential phase, and 96 h incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 h incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hours of incubation (Fig. 6bc), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hours of incubation. Decreased in surface tension values are shown in Fig. 6d.

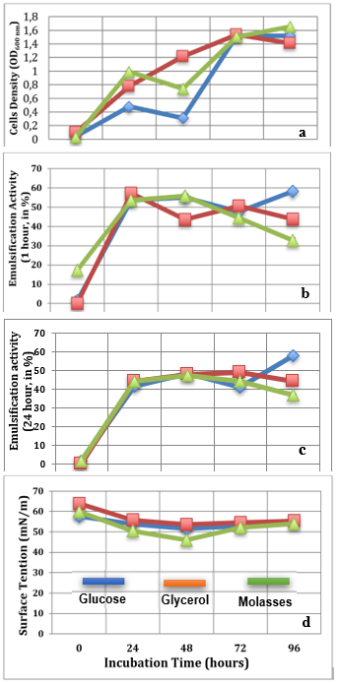

Fig. 6 Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: **a** cells density, **b** emulsification activity (1 hour), **c** emulsification activity (24 hours), **d** surface tension value

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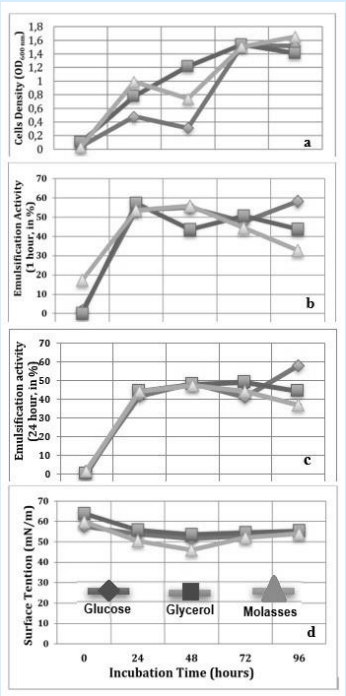

Discussions

The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Fig. 1). Bacillus sp. BK7.1 has a 98.68% similarity to Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 based on molecular identification.~~Based on molecular identification, *Bacillus* sp. BK7.1, which similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28, homology level of 98.68%.~~ The gene of 16S rRNA can be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing is a fast and accurate method for bacterial identification. Bacteria represent the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019, Srinivasan et al. 2015). The similarity is less than 100% because there are variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research has shown~~conducted~~ that *B. subtilis* strain SBMP4 can control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted to and grown in extreme environmental conditions, forms endospores that are resistant to stress, and secretes~~has secreted~~ various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic is the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016, Mishra and Arora 2018). Surfactin produced by *B. subtilis* is one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013, Gudina et al. 2016).

Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain can kill adult mosquitoes. The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity occurs through two different mechanisms,

at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes it will causes osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria can produce biosurfactants if they can reduce surface tension values by ≥ 10 mN/m (de Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* is caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 causes the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin can be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (Guimarães et al. 2019).

The emulsification index value of *B. subtilis* BK7.1 is a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure causes surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin are involved in cell attachment and cause membrane disruption (Chen et al. 2022). The ability of surfactin to bind Ca^{2+} causes a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Khedher et al. 2015, Khedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation tends to decrease compared to 24 hours observation. This difference has shown that the emulsion is unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, are efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, are more effective in stabilizing oil-in-water emulsions as emulsifiers (Uzoigwe et al. 2015). This result is supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affect the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species have srfAA gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (Plaza et al. 2015). The nonribosomal peptide synthetase complex is coded by srfAA and srfAD gene known as surfactin synthetase. The srfAA and srfAD genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase is an activating enzyme for the srfA multienzyme complex. The srfAA, srfAB, srfAC, and srfAD genes are involved in the assembly of heptamodular non-ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene transforms surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by srfAA and srfAD gene (Plaza et al. 2015). The Table 3 showed that the similarity results have a value of 91.04%, because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity can cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. The higher emulsification activity from *B. subtilis* 573 to 48.4% (Pereira et al. 2013), *B. subtilis* 21332 up to 55.2% (Zhu et al. 2016), and *B. subtilis* N3-4P up to 38.3% (Zhu et al. 2016) on mineral salt media containing using different carbon sources than glycerol. The production of biosurfactant by *Bacillus nealsonii* S2M in glycerol substrate has been able to emulsify various hydrocarbons in 55% (Phulpoto et al. 2020).

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Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al.

2014). The difference in surface tension reduction is caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide sequences between bacteria species affect the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate is influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. inaquosorum strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The srfAA and srfAD genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (Penelitian Unggulan Fakultas) 2022-2023. The authors grate full to the Dean of the Faculty of Science and Technology (Fakultas Sains dan Teknologi) and Head of Research and Community Services Institutions (Lembaga Penelitian dan Pengabdian Masyarakat), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia

Abstract. Biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research aimed to identify the species and genetic relationship, hemolytic activity, detect coding genes, and trial production of biosurfactants on various substrates of entomopathogenic *Bacillus* sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Biosurfactant screening was carried out by testing hemolytic activity, surface tension, and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing biosurfactant production in various substrates. The results of the molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method for *Bacillus* sp. BK7.1 has a genetic similarity of 98.68% with *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension, increased emulsification activities, and the production of biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that *Bacillus* sp. BK7.1 had srfAA and srfAD genes encoding surfactin biosynthesis, giving it the potential to produce bioinsecticide compounds. Based on these studies, the indigenous entomopathogenic *B. subtilis* BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

Keywords: Biosurfactant production, crop protection, entomopathogenic *Bacillus subtilis* BK7.1, hemolytic activity, srfAA-srfAD gene.

Running Title: Biosurfactant production of *Bacillus subtilis* BK7.1

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is used widely all around the globe (Safni et al. 2018). However, the chemical insecticides have a negative impact on control of disease vector and pest because they cause insect resistance (Şengül et al. 2022). There are a number of biocontrol methods available to resolve these problems. Entomopathogens are natural enemies that can produce toxic metabolites against insect pests and plant pathogens. Biocontrol methods can be used as an alternative in fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. These methods do not cause pollution and are environmentally friendly (Thomas 2017).

Biocontrol agents using *Bacillus* strains are methods that have been widely developed because they are proven to be environmentally friendly (Abdel-Aziz et al. 2020, Bergamasco et al. 2013, Syaharuddin et al. 2018, Qureshi et al. 2021).

A group of bacteria, fungi, and yeasts have produced biosurfactants are capable of producing biosurfactants with different surface activities and molecular structures (Santos et al. 2018). Several groups of microbes can synthesize biosurfactants, which can be used to replace non-biodegradable and non-environmental friendly synthetic surfactants (Moro et al. 2018). The biosurfactant produced by *Bacillus* is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features including high levels of biodegradability and optimal activity under extreme conditions (Khedher et al. 2017). Following previous studies, *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. velezensis* produce biosurfactant and are efficient biocontrol agents against different targets (Revathi et al. 2013, Nafidiastri et al. 2021). *Bacillus* sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (Théatre et al. 2021). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and triggering systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016, Pele et al. 2019, Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016, Chaves et al. 2018, Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature conditions often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic *Bacillus* spp. are still being carried out to find the safest way to control disease vectors transmitted ~~caused by~~ mosquitoes. The results of screening tests for potential initial toxicity against *A. aegypti* larvae have reported that 68 entomopathogenic *Bacillus* sp. which have been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). *Bacillus* sp. BK5.2, after molecular identification, has been identified as *B. thuringiensis* BK5.2 which produces an entomopathogenic cry toxin (Salamun et al. 2021). The identification of *Bacillus* sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of *Bacillus* sp. BK7.1 as an entomopathogenic bacteria. Genetic characteristics were used in this study to determine the species and their relationships in the phylogenetic tree, as well as the detection of biosurfactant coding genes and the screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on various substrates.

MATERIALS AND METHODS

Bacteria and Culture Condition

Bacillus sp. BK7.1 was isolated from Baluran National Park soil samples. This isolate has already been identified conventionally such as macroscopic, microscopic, and physiological, was identified as *B. sphaericus*, but further identification is needed through 16S rRNA in an effort development of future research like bioinsectiside product (Salamun et al. 2020). This bacteria was maintained aerobically on NB agar plates and was regularly transferred into fresh NB medium slant for shortterm storage.

Identification 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r), examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and visualized under ultraviolet light, then purified and sequenced. Amplicon result was then aligned and contigs were

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developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with Du Nouy Tensiometer, with 50% Tween 20 as a positive control and Nutrient Broth as a negative control. The decrease in the surface tension value (10 mN/m) indicated the potential to produce biosurfactants. The emulsification activity was measured by inserting a 2 mL supernatant fraction and kerosene in a test tube. This mixture was stirred on Vortex Mixer for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection srfAA and srfAD surfactin gene

Amplification of the srfAA and srfAD surfactin genes of *Bacillus* sp. BK7.1 was carried out by using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3' and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for srfAA gene (Mora et al. 2020, Kim et al. 2020). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATTGGATGCT 3' for srfAD gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

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Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolving one by one, 3 g (NH4)2SO4, 10 g NaCl, 0.2 g MgSO4.7H2O, 0.01 g CaCl2, 0.001 g MnSO4.H2O, 0.001 g H3BO3, 0.001 g ZnSO4.7H2O, 0.001 g CuSO4.5H2O, 0.005 g CoCl2.6H2O, and 0.001 g NaMoO4.2H2O into 900 mL distilled water, respectively. The elements phosphate and iron were made separately. The phosphate elements dissolved 5 g of KH2PO4 and 2 g of K2HPO4 into 50 mL of distilled water, while, the iron element dissolved 0.0006 g of FeSO4.7H2O into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hrs with an agitation of 130 rpm. Every 24 hrs, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hrs incubation.

RESULTS AND DISCUSSION

Results

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Fig. 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28, homology level of 98.68% (Table 1).

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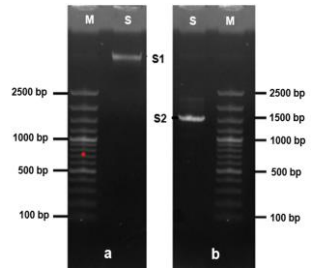

Fig. 1 The electrophoresis results of DNA genome (**a**) and 16S rRNA gene (**b**) of *Bacillus* sp. BK7.1 on 1% agarose gel. Descriptions: **M** 100 bp DNA marker, **S** sample, **S1** sample of DNA genome, **S2** sample of 16SrRNA gene

Table 1 The species of *Bacillus* sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

No.	Species	Accession No.	E value	%ID	Query Cover (%)
1	*Bacillus subtilis* subsp. inaquosorum strain BGSC 3A28	NR_104873.1	0.0	98.68	99
2	*Bacillus subtilis* strain DSM 10	NR_027552.1	0.0	98.61	99
3	*Bacillus subtilis* strain JCM 1465	NR_113265.1	0.0	98.61	99

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Fig. 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

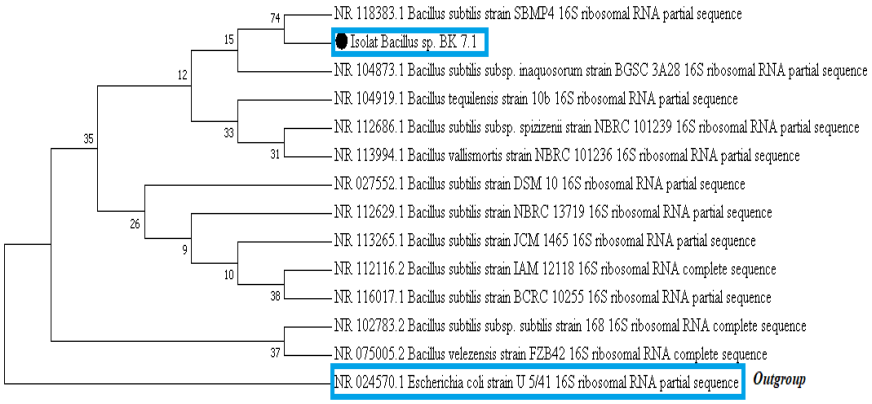

Fig. 2 Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Fig. 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83

mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which was left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it was decreased by 21.92% (Fig. 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

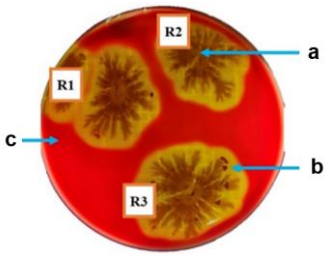

Fig. 3 Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. Descriptions: **a** isolate, **b** clear zone around the colony, **c** blood agar plate, **R** Replicates.

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

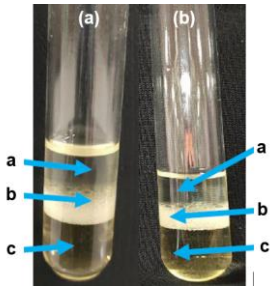

Fig. 4 The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. Descriptions: **a** kerosene, **b** emulsion, **c** isolate, (**a**) 1 hour of exposure, (**b**) 24 hours of exposure.

Detection srfAA and srfAD surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as srfAA gene, and 723 bp expected as srfAD gene (Fig. 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

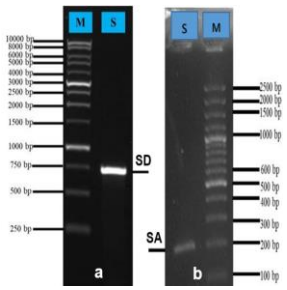

Fig. 5 The electrophoresis results of srfAD (**a**) and srfAA (**b**) surfactin gene amplification of *Bacillus subtilis* BK7.1. Description: **M** 100 bp DNA marker, **SA** sample of srfAA surfactin gene 201 bp, **SD** sample of srfAD surfactin gene 729 bp

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of srfAA and srfAD protein isolates of *Bacillus subtilis* BK 7.1

No.	Protein	Species	Accession No.	E value	%ID	Query Cover (%)
1.	surfactin non-ribosomal peptide synthetase srfAA	*Bacillus subtilis inaquosorum*	WP_060397903.1	9e-34	91.04	100
2.	surfactin biosynthesis thioesterase SrfAD	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

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Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Fig. 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Fig. 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 hrs incubation, isolates still showed an exponential phase, and 96 hrs incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 hrs incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hrs of incubation (Fig. 6bc), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hrs of incubation. Decreased in surface tension values are shown in Fig. 6d.

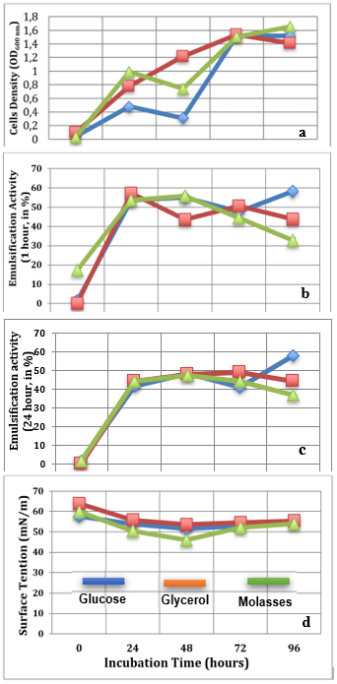

Fig. 6 Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: **a** cells density, **b** emulsification activity (1 hour), **c** emulsification activity (24 hours), **d** surface tension value

Discussions

Conventional identification of *Bacillus* sp. BK7.1 has been carried out. Based on the macroscopic, microscopic, and physiological characteristics of *Bacillus* sp. BK7.1 has similarities with *Bacillus sphaericus* (Salamun et al., 2020). Researchers suggest further research to confirm the species name, by identifying the 16S rRNA gene. The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Fig. 1). *Bacillus* sp. BK7.1 had ~~has~~ a 98.68% similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28 based on molecular identification. The gene of 16S rRNA ~~can~~ could be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing ~~is~~ was a fast and accurate method for bacterial identification. Bacteria represented the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019, Srinivasan et al. 2015). The similarity was ~~is~~ less than 100% because there ~~are~~ were variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research has shown that *B. subtilis* strain SBMP4 ~~can~~ could control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted to and grown in extreme environmental conditions, forms endospores that are resistant to stress, and secretes various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic ~~is~~ was the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016, Mishra and Arora 2018). Biosurfactant lipopeptides from entomopathogenic microbes ~~can~~ could act as biocontrol, especially antimicrobials and anti-biofilms (Abdel-Aziz et al. 2020, Qureshi et al. 2021). Surfactin produced by *B. subtilis* ~~is~~ was one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013, Gudina et al. 2016).

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Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain ~~can~~ could kill adult mosquitoes. The hemolytic activity of *B. subtilis* BK7.1 ~~can~~ could be seen in Fig. 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity ~~occurs~~ occured through two different mechanisms, at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes and cause osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria ~~can~~ could produce biosurfactants if they can reduce surface tension values by ≥ 10 mN/m (de Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* ~~is~~ was caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 ~~causes~~ caused the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin ~~can~~ could be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (Guimarães et al. 2019).

The emulsification index value of *B. subtilis* BK7.1 ~~is~~ was a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure ~~causes~~ caused surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin ~~are~~ were involved in cell attachment and cause membrane disruption (Chen et al. 2022). The ability of surfactin to bind Ca^{2+} ~~causes~~ caused a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Khedher et al. 2015, Khedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation ~~tends~~ tended to decrease compared to 24 hrs observation. This difference has shown that the emulsion ~~is~~ was unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, ~~are~~ were efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, ~~are~~ were more effective in stabilizing oil-in-water emulsions as emulsifiers (Uzoigwe et al. 2015). This result ~~is~~ was supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affected the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species ~~have~~ had srfAA gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (Plaza et al. 2015). The nonribosomal peptide synthetase complex ~~is~~ was coded by srfAA and srfAD gene known as surfactin synthetase. The srfAA and srfAD genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase ~~is~~ was an activating enzyme for the srfA multienzyme complex. The srfAA, srfAB, srfAC, and srfAD genes ~~are~~ were involved in the assembly of heptamodular non-ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene ~~transforms~~ transformed surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by srfAA and srfAD gene (Plaza et al. 2015). The Table 3 showed that the similarity results have a value of 91.04%, because there ~~are~~ were several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity ~~can~~ could cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. The higher emulsification activity from *B. subtilis* 573 to 48.4% (Pereira et al. 2013), *B. subtilis* 21332 up to 55.2% (Zhu et al. 2016), and *B. subtilis* N3-4P up to 38.3% (Zhu et al. 2016) on mineral salt media containing using different carbon sources than glycerol. The production of biosurfactant by *Bacillus nealsonii* S2M in glycerol substrate has been able to emulsify various hydrocarbons in 55% (Phulpoto et al. 2020).

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Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al. 2014). The difference in surface tension reduction ~~is~~ was caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide sequences between bacteria species affected the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate ~~is~~ was influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The srfAA and srfAD genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health. Therefore, this research needs to be followed up to detect the chemical components of biosurfactants produced by these bacteria.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (Penelitian Unggulan Fakultas) 2022-2023. The authors grate full to the Dean of the Faculty of Science and Technology (Fakultas Sains dan Teknologi) and Head of Research and Community Services Institutions (Lembaga Penelitian dan Pengabdian Masyarakat), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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Round 5

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BIODIVERSITAS
Volume 24, Number 3, March 2023
Pages: xxxx

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d2403xx

Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia

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Manuscript received: 27 January 2023. Revision accepted: xxx February 2023.

Abstract. *Salamun, Susetyo RD, Ni'matuzahroh, Fatimah, Geraldi A, Supriyanto A, Nurhariyati T, Nafidiastri FA, Nisa N, Endarto. 2023. Biosurfactant production of entomopathogenic* Bacillus subtilis *BK7.1, as potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia. Biodiversitas 24: xxx.* Biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research aimed to identify the species and genetic relationship, hemolytic activity, detect coding genes, and trial production of biosurfactants on various substrates of entomopathogenic *Bacillus* sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Biosurfactant screening was carried out by testing hemolytic activity, surface tension, and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing biosurfactant production in various substrates. The results of the molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method for *Bacillus* sp. BK7.1 has a genetic similarity of 98.68% with *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension, increased emulsification activities, and the production of biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that *Bacillus* sp. BK7.1 had srfAA and srfAD genes encoding surfactin biosynthesis, giving it the potential to produce bioinsecticide compounds. Based on these studies, the indigenous entomopathogenic *B. subtilis* BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

Keywords: Biosurfactant production, crop protection, entomopathogenic *Bacillus subtilis* BK7.1, hemolytic activity, srfAA-srfAD gene.

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is used widely all around the globe (Safni et al. 2018). However, the chemical insecticides have a negative impact on control of disease vector and pest because they cause insect resistance (Şengül et al. 2022). There are a number of biocontrol methods available to resolve these problems. Entomopathogens are natural enemies that can produce toxic metabolites against insect pests and plant pathogens. Biocontrol methods can be used as an alternative in fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. These methods do not cause pollution and are environmentally friendly (Thomas 2017).

Biocontrol agents using *Bacillus* strains are methods that have been widely developed because they are proven to be environmentally friendly (Bergamasco et al. 2013; Syaharuddin et al. 2018; Abdel-Aziz et al. 2020; Qureshi et al. 2021). A group of bacteria, fungi, and yeasts have produced biosurfactants are capable of producing biosurfactants with different surface activities and molecular structures (Santos et al. 2018). Several groups of microbes can synthesize biosurfactants, which can be used to replace non-biodegradable and non-environmental friendly synthetic surfactants (Moro et al. 2018). The biosurfactant produced by *Bacillus* is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites

that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features including high levels of biodegradability and optimal activity under extreme conditions (Khedher et al. 2017). Following previous studies, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus velezensis* produce biosurfactant and are efficient biocontrol agents against different targets (Revathi et al. 2013; Nafidiastri et al. 2021). *Bacillus* sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (Théatre et al. 2021). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and triggering systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016, Pele et al. 2019, Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016; Martins and Martins et al. 2018; Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature conditions often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic *Bacillus* spp. are still being carried out to find the safest way to control disease vectors transmitted by mosquitoes. The results of screening tests for potential initial toxicity against *Aedes aegypti* Linnaeus, 1762 larvae have reported that 68 entomopathogenic *Bacillus* sp. which have been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). *Bacillus* sp. BK5.2, after molecular identification, has been identified as *Bacillus thuringiensis* BK5.2 which produces an entomopathogenic cry toxin (Salamun et al. 2021). The identification of *Bacillus* sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of *Bacillus* sp. BK7.1 as an entomopathogenic bacteria. Genetic characteristics were used in this study to determine the species and their relationships in the phylogenetic tree, as well as the detection of biosurfactant coding genes and the screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on various substrates.

MATERIALS AND METHODS

Isolation and identification of bacteria

Bacillus sp. BK7.1 was isolated from Baluran National Park soil samples. This isolate was identified conventionally such as macroscopic, microscopic, and physiological characters first and then at molecular level through 16S rRNA (Salamun et al. 2020). This bacteria was maintained aerobically on NB agar plates and was regularly transferred into fresh NB medium slant for short-term storage.

Molecular identification using 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r) was examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and visualized under ultraviolet light, then purified and sequenced. Amplicon results were then aligned and contigs were developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with du nouy tensiometer, with 50% tween 20 as a positive control and nutrient broth as a negative control. The decrease in the surface tension value (10 mN/m) indicated the potential to produce biosurfactants. The emulsification activity was measured by inserting a 2mL supernatant fraction and kerosene in a test tube. This mixture was stirred on vortex mixer for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection srfAA and srfAD surfactin gene

Amplification of the srfAA and srfAD surfactin genes of *Bacillus* sp. BK7.1 was carried out by using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3' and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for srfAA gene (Mora et al. 2020, Kim et al. 2020). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATTGGATGCT 3' for srfAD gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolving one by one, 3 g (NH4)2SO4, 10 g NaCl, 0.2 g MgSO4.7H2O, 0.01 g CaCl2, 0.001 g MnSO4.H2O, 0.001 g H3BO3, 0.001 g ZnSO4.7H2O, 0.001 g CuSO4.5H2O, 0.005 g CoCl2.6H2O, and 0.001 g NaMoO4.2H2O into 900 mL distilled water, respectively. The elements phosphate and iron were made separately. The phosphate elements dissolved 5 g of KH2PO4 and 2 g of K2HPO4 into 50 mL of distilled water, while, the iron element dissolved 0.0006 g of FeSO4.7H2O into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hrs with an agitation of 130 rpm. Every 24 hrs, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hrs incubation.

RESULTS AND DISCUSSION

Results

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Figure 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28, homology level of 98.68% (Table 1).

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Figure 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Figure 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83 mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which was left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it was decreased by 21.92% (Figure 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

Table 1. The species of Bacillus sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

Species	Accession No.	E value	%ID	Query Cover (%)
Bacillus subtilis subsp. *inaquosorum strain* BGSC 3A28	NR_104873.1	0.0	98.68	99
Bacillus subtilis strain DSM 10	NR_027552.1	0.0	98.61	99
Bacillus subtilis strain JCM 1465	NR_113265.1	0.0	98.61	99

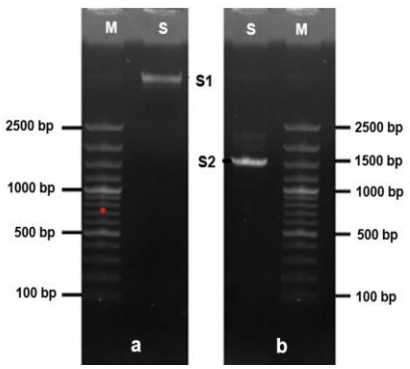

Figure 1. The electrophoresis results of DNA genome (a) and 16S rRNA gene (b) of Bacillus sp. BK7.1 on 1% agarose gel. Descriptions: M 100 bp DNA marker, S sample, S1 sample of DNA genome, S2 sample of 16SrRNA gene

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

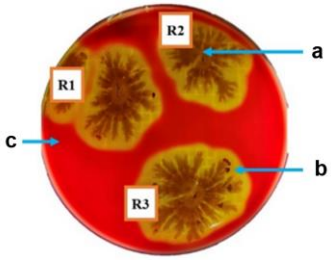

Figure 3. Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. Descriptions: a isolate, b clear zone around the colony, c blood agar plate, R Replicates.

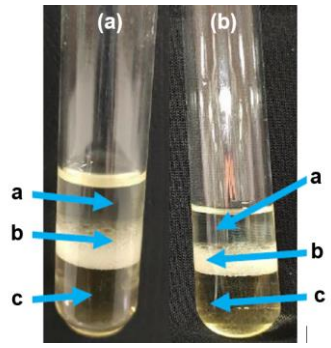

Figure 4. The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. Descriptions: a kerosene, b emulsion, c isolate, (a) 1 hour of exposure, (b) 24 hours of exposure.

Detection srfAA and srfAD surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as srfAA gene, and 723 bp expected as srfAD gene (Figure 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Figure 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Fig. 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 hrs incubation, isolates still showed an exponential phase, and 96 hrs incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 hrs incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hrs of incubation (Figure 6b and Figure 6c), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hrs of incubation. Decreased in surface tension values are shown in Figure 6d.

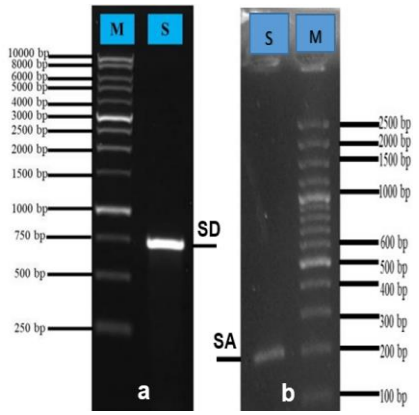

Figure 5. The electrophoresis results of srfAD (a) and srfAA (b) surfactin gene amplification of Bacillus subtilis BK7.1. Description: M 100 bp DNA marker, SA sample of srfAA surfactin gene 201 bp, SD sample of srfAD surfactin gene 729 bp

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of srfAA and srfAD protein isolates of *Bacillus subtilis* BK 7.1

No.	Protein	Species	Accession No.	E value	%ID	Query Cover (%)
1.	surfactin non-ribosomal peptide synthetase srfAA	*Bacillus subtilis inaquosorum*	WP_060397903.1	9e-34	91.04	100
2.	surfactin biosynthesis thioesterase SrfAD	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

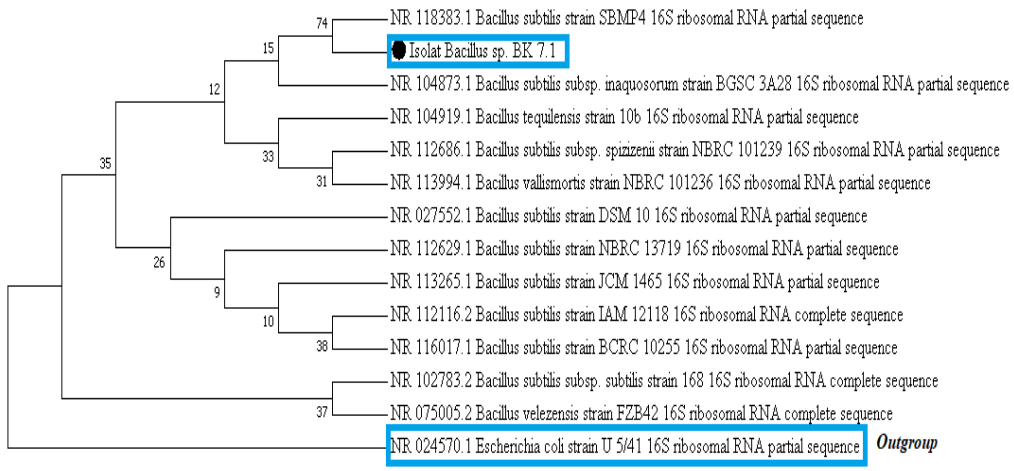

Figure 2. Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species

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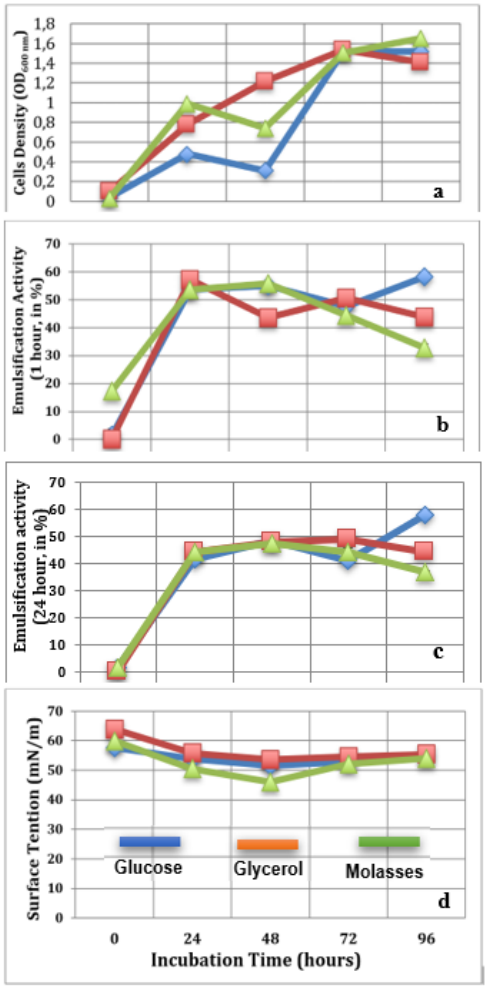

Figure 6. Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: a cells density, b emulsification activity (1 hour), c emulsification activity (24 hours), d surface tension value

Discussions

Conventional identification of *Bacillus* sp. BK7.1 has been carried out. Based on the macroscopic, microscopic, and physiological characteristics of *Bacillus* sp. BK7.1 has similarities with *Bacillus sphaericus* (Salamun et al., 2020). Researchers suggest further research to confirm the species name, by identifying the 16S rRNA gene. The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Figure 1). *Bacillus* sp. BK7.1 had a 98.68% similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28 based on molecular identification. The gene of 16S rRNA could be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing is was a fast and accurate method for bacterial identification. Bacteria represented the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019; Srinivasan et al. 2015). The similarity was less than 100% because there were variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research has shown that *B. subtilis* strain SBMP4 could control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted to and grown in extreme environmental conditions, forms endospores that are resistant to stress, and secretes various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic was the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016; Mishra and Arora 2018). Biosurfactant lipopeptides from entomopathogenic microbes could act as biocontrol, especially antimicrobials and anti-biofilms (Abdel-Aziz et al. 2020, Qureshi et al. 2021). Surfactin produced by *B. subtilis* was one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration

of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013; Gudina et al. 2015).

Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain could kill adult mosquitoes. The hemolytic activity of *B. subtilis* BK7.1 could be seen in Figure 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity occurred through two different mechanisms, at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes and cause osmotic lysis (Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria could produce biosurfactants if they can reduce surface tension values by ≥10 mN/m (Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* was caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 caused the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin could be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (Guimarães et al. 2019).

The emulsification index value of *B. subtilis* BK7.1 was a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure caused surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin were involved in cell attachment and cause membrane disruption (Chen et al. 2022). The ability of surfactin to bind Ca^{2+} caused a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Khedher et al. 2015, Khedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation tended to decrease compared to 24 hrs observation. This difference has shown that the emulsion was unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, were efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, were more effective in stabilizing oil-in-water emulsions as emulsifiers (Uzoigwe et al. 2015). This result was supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affected the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species had srfAA gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (Plaza et al. 2015). The nonribosomal peptide synthetase complex was coded by srfAA and srfAD gene known as surfactin synthetase. The srfAA and srfAD genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase was an activating enzyme for the srfA multienzyme complex. The srfAA, srfAB, srfAC, and srfAD genes were involved in the assembly of heptamodular non-ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene transformed surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by srfAA and srfAD gene (Plaza et al. 2015). The Table 3 showed that the similarity results have a value of 91.04%, because there were several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity could cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. The higher emulsification activity from *B. subtilis* 573 to 48.4% (Pereira et al. 2013), *B. subtilis* 21332 up to 55.2% (Zhu et al. 2016), and *B. subtilis* N3-4P up to 38.3% (Zhu et al. 2016) on mineral salt media containing using different carbon sources than glycerol. The production of biosurfactant by *Bacillus nealsonii* S2M in glycerol substrate has been able to emulsify various hydrocarbons in 55% (Phulpoto et al. 2020).

Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al. 2014). The difference in surface tension reduction was caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide

sequences between bacteria species affected the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate was influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The srfAA and srfAD genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health. Therefore, this research needs to be followed up to detect the chemical components of biosurfactants produced by these bacteria.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (Penelitian Unggulan Fakultas) 2022-2023 and Featured Basic Research (Penelitian Dasar Unggulan) 2023 Universitas Airlangga. The authors grate full to the Dean of the Faculty of Science and Technology (Fakultas Sains dan Teknologi) and Head of Research and Community Services Institutions (Lembaga Penelitian dan Pengabdian Masyarakat), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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BIODIVERSITAS
Volume 24, Number 3, March 2023
Pages: 1785-1792

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240353

Biosurfactant production of entomopathogenic *Bacillus subtilis* BK7.1, as potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia

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Manuscript received: 27 January 2023. Revision accepted: 25 March 2023.

Abstract. *Salamun, Susetyo RD, Ni'matuzahroh, Fatimah, Geraldi A, Supriyanto A, Nurhariyati T, Nafidiastri FA, Nisa' N, Endarto. 2023. Biosurfactant production of entomopathogenic* Bacillus subtilis *BK7.1, as potential biocontrol bacteria, isolated from Baluran National Park, East Java, Indonesia. Biodiversitas 24: 1785-1792.* Biosurfactants as biocontrol agents have received much attention for pest control and disease vectors. The research aimed to identify the species and genetic relationship, hemolytic activity, detect coding genes, and trial production of biosurfactants on various substrates of entomopathogenic *Bacillus* sp. BK7.1 isolated from natural soil in Baluran National Park, East Java, Indonesia. Biosurfactant screening was carried out by testing hemolytic activity, surface tension, and emulsification activities, detecting coding genes of biosurfactant biosynthesis, and testing biosurfactant production in various substrates. The results of the molecular identification by amplifying the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method for *Bacillus* sp. BK7.1 has a genetic similarity of 98.68% with *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. Screening showed positive hemolytic activity results, reduced surface tension, increased emulsification activities, and the production of biosurfactant in glucose, glycerol, and molasses substrates. The PCR results showed that *Bacillus* sp. BK7.1 had *srfAA* and *srfAD* genes encoding surfactin biosynthesis, giving it the potential to produce bioinsecticide compounds. Based on these studies, the indigenous entomopathogenic *B. subtilis* BK7.1 can be developed as environmentally friendly microbial bioinsecticides for pest control and disease vectors.

Keywords: *Bacillus subtilis* BK7.1, biosurfactant production, crop protection, entomopathogenic, hemolytic activity, *srfAA-srfAD* gene

INTRODUCTION

Controlling insect pests and insect vectors with chemical insecticides is used widely all around the globe (Safni et al. 2018). However, the chemical insecticides have a negative impact on control of disease vector and pest because they cause insect resistance (Şengül et al. 2022). There are a number of biocontrol methods available to resolve these problems. Entomopathogens are natural enemies that can produce toxic metabolites against insect pests and plant pathogens. Biocontrol methods can be used as an alternative in fighting diseases transmitted by vector mosquitoes, plant pathogens, and insect pests. These methods do not cause pollution and are environmentally friendly (Thomas 2017).

Biocontrol agents using *Bacillus* strains are methods that have been widely developed because they are proven to be environmentally friendly (Bergamasco et al. 2013; Syaharuddin et al. 2018; Abdel-Aziz et al. 2020; Qureshi et al. 2021). A group of bacteria, fungi, and yeasts have produced biosurfactants are capable of producing biosurfactants with different surface activities and molecular structures (Santos et al. 2018). Several groups of microbes can synthesize biosurfactants, which can be used to replace non-biodegradable and non-environmental friendly synthetic surfactants (Moro et al. 2018). The biosurfactant produced by *Bacillus* is one of the entomopathogenic mechanisms that have caused the death of insects. Biosurfactants are unique microbial metabolites that appear in biological action against plant pathogens and insect pests.

Biosurfactants have many interesting features including high levels of biodegradability and optimal activity under extreme conditions (Khedher et al. 2017). Following previous studies, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus velezensis* produce biosurfactant and are efficient biocontrol agents against different targets (Revathi et al. 2013; Nafidiastri et al. 2021). *Bacillus* sp. is able to synthesize lipopeptide biosurfactants, such as surfactin, fengicin, and iturin (Théatre et al. 2021). Surfactin consists of 7 amino acids bonded to a carboxyl group and a fatty acid hydroxyl group at carbon atoms number 12-16, synthesized by a complex mechanism, catalyzed by Nonribosomal Peptide Synthetase (NRPS) and encoded by the srfA operon. Surfactin can suppress plant diseases through strong biosurfactant activity (Cawoy et al. 2014) by inhibiting bacterial growth, lysing cell membranes or destroying them through physicochemical interactions (Deleu et al. 2013), suppressing fungi by promoting colonization of beneficial bacteria (Jia et al. 2015), and triggering systemic resistance (Cawoy et al. 2014). Biosurfactants have been applied in various industrial and petroleum fields (Nwaguma et al. 2016; Pele et al. 2019; Gomaa et al. 2019). Biosurfactants are lower in toxicity, more biodegradable and environmentally friendly, harmless, and work more specifically (De Almeida et al. 2016; Martins and Martins et al. 2018; Gayathiri et al. 2022). Biosurfactants are stable and efficient under unfavorable salinity, pH and temperature conditions often encountered in the petroleum industry (Silva et al. 2014). Biosurfactants can reduce surface and interfacial tension, as well as suitable emulsifiers and dispersing agents and are widely used in the industrial sector (Mulligan et al. 2014).

Perspective studies to find entomopathogenic *Bacillus* spp. are still being carried out to find the safest way to control disease vectors transmitted by mosquitoes. The results of screening tests for potential initial toxicity against *Aedes aegypti* Linnaeus, 1762 larvae have reported that 68 entomopathogenic *Bacillus* sp. which have been isolated from 30 natural soil samples with potential status variations from low to very high. In the affirmation test, there were three isolates coded BK7.1, BK7.2, and BK5.2, with the highest entomopathogenic potential status, larval mortality rates at 48 hours of exposure were 93, 87 and 70%, respectively (Salamun et al. 2020). *Bacillus* sp. BK5.2, after molecular identification, has been identified as *Bacillus thuringiensis* BK5.2 which produces an entomopathogenic cry toxin (Salamun et al. 2021). The identification of *Bacillus* sp. BK7.1 has been carried out through morphological and physiological characterization (Salamun et al. 2020). It is necessary to carry out molecular identification and mechanism of action of *Bacillus* sp. BK7.1 as an entomopathogenic bacteria. Genetic characteristics were used in this study to determine the species and their relationships in the phylogenetic tree, as well as the detection of biosurfactant coding genes and the screening of biosurfactant activities such as hemolytic activity, surface tension, emulsification activity, and production on various substrates.

MATERIALS AND METHODS

Isolation and identification of bacteria

Bacillus sp. BK7.1 was isolated from Baluran National Park soil samples. This isolate was identified conventionally such as macroscopic, microscopic, and physiological characters first and then at molecular level through 16S rRNA (Salamun et al. 2020). This bacteria was maintained aerobically on NB agar plates and was regularly transferred into fresh NB medium slant for short-term storage.

Molecular identification using 16 S rRNA gene

The DNA genome of *Bacillus* sp. BK7.1 was isolated according to the Thermo Scientific GeneJet Genomic DNA Purification Kit, visualized under ultraviolet by electrophoresis, purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer; purity was calculated by the ratio between the values of 260 nm and 280 nm in the DNA samples (Meena et al. 2020). Amplifying genomic DNA of *Bacillus* sp. BK7.1 utilized 16S rRNA primers (27f and 1492r) was examined by electrophoresis on 1% agarose gel followed by ethidium bromide (EtBr) dye and visualized under ultraviolet light, then purified and sequenced. Amplicon results were then aligned and contigs were developed from the sequences using the BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA nucleotide sequence was aligned with 16S rRNA gene sequences from other microorganisms published in GenBank. Genetic similarity was determined to contig alignment and phylogenetic tree construction using the Program of Mega 7. The phylogenetic tree was designed by inputting FASTAs from BLAST species (Kumar et al. 2016).

Screening biosurfactant activities

Screening of biosurfactants was carried out by three methods, hemolytic activity, surface tension value, and emulsification activity. Hemolytic activity using blood agar media inoculated with *Bacillus* sp. BK7.1 by spots method and incubated for two days at room temperature and zone of inhibition observed around the colony. Surface tension was measured with *du nouy* tensiometer, with 50% tween 20 as a positive control and nutrient broth as a negative control. The decrease in the surface tension value (10 mN/m) indicated the potential to produce biosurfactants. The emulsification activity was measured by inserting a 2 mL supernatant fraction and kerosene in a test tube. This mixture was stirred on vortex mixer for 1 minute, incubated for 24 hours at room temperature, and measured after the emulsion height was stable. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E24).

Detection *srfAA* and *srfAD* surfactin gene

Amplification of the *srfAA* and *srfAD* surfactin genes of *Bacillus* sp. BK7.1 was carried out by using primers selected according to the literature. Electrophoresis and visualization were performed under UV Transluminator. Forward primer F-5' TCGGGACAGGAAGACATCAT 3'

and reverse primer R-5' CCACTCAAACGGATAATCCTGA 3' for *srfAA* gene (Mora et al. 2020; Kim et al. 2020). Forward primer F-5' ATGAGCCAACTCTTCAAATCATTTG 3' and reverse primer R-5' TCACGATTGAATGATT GGATGCT 3' for *srfAD* gene. The amplicons were aligned and developed from the sequences by the BioEdit Sequence Alignment Editor for Windows software. The nucleotide sequences are translated into a protein to be formed. The translation of the nucleotide sequence aligned with BLASTp from the other *Bacillus*, which has been published on GenBank.

Biosurfactant production

The biosurfactant production activity begins by providing synthetic mineral water (SMW), by dissolving one by one, 3 g $(NH_4)2SO_4$, 10 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2$, 0.001 g $MnSO_4.H_2O$, 0.001 g H_3BO_3, 0.001 g $ZnSO_4.7H_2O$, 0.001 g $CuSO_4.5H_2O$, 0.005 g $CoCl_2.6H_2O$, and 0.001 g $NaMoO_4.2H_2O$ into 900 mL distilled water, respectively. The elements phosphate and iron were made separately. The phosphate elements dissolved 5 g of KH_2PO_4 and 2 g of K_2HPO_4 into 50 mL of distilled water, while the iron element dissolved 0.0006 g of $FeSO_4.7H_2O$ into 50 mL of distilled water, respectively. The phosphate and iron elements were sterilized using an autoclave for 15-20 minutes at 121°C with 1 atm.

A 250 mL culture bottle was prepared to be filled with 86.4 mL of SMW and added 2% substrates (glucose, glycerol, molasses) solution, was homogenized and ensured that the pH was 7.0. The culture vial was sterilized by autoclave for 15-20 minutes at 121°C 1 atm. After sterilization, the culture was cooled at room temperature, then 4.8 mL of phosphate and iron elements were added. Then each added 4% (4 mL) of bacterial culture with an absorbance value of 0.5 Optical Density in 650 nm. The culture solution was incubated at room temperature for 96 hrs with an agitation of 130 rpm. Every 24 hrs, bacterial biomass, surface tension value, and emulsification activity against diesel and kerosene were measured until 96 hrs incubation.

RESULTS AND DISCUSSION

Identification of 16S rRNA gene

Purity and concentration of DNA genome of *Bacillus* sp. BK 7.1 obtained a 1.782 and a 31 ng/µL and after being confirmed with agarose gel electrophoresis 1% in Figure 1. *Bacillus* sp. BK7.1 has a size of 1449 bp of the 16S rRNA nucleotide sequence, which similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28, homology level of 98.68% (Table 1).

The phylogenetic analysis results where *Bacillus* sp. BK7.1 and some strain of known *Bacillus* are presented in Figure 2. The closest relative of *Bacillus* sp. BK7.1 is a strain of *Bacillus subtilis* strain SBMP4, and this grouping only shows the closeness of the strains based on the similarity of the 16S rRNA sequence, and does not describe the ability to produce biosurfactants, especially surfactin.

Screening of biosurfactant activity

The hemolytic activity of *B. subtilis* BK7.1 can be seen in Figure 3. The surface tension value of the supernatant fraction of *B. subtilis* BK7.1 of 49.17 mN/m can be seen in Table 2. When compared with the surface tension value of the control in the form of distilled water and the control media of Nutrient Broth (NB), the value of the culture supernatant of *B. subtilis* BK7.1 experienced a decrease in surface tension value of 15.21 mN/m from the NB media control and 22.83 mN/m from the distilled water control. The emulsification index value of the supernatant *B. subtilis* BK7.1 of 18.02%, which was left for one hour while after 24 hours the emulsification index value becomes 25.53%, where it was decreased by 21.92% (Figure 4). The emulsification index value indicates the stability of the emulsion and lines that produce values above 50%.

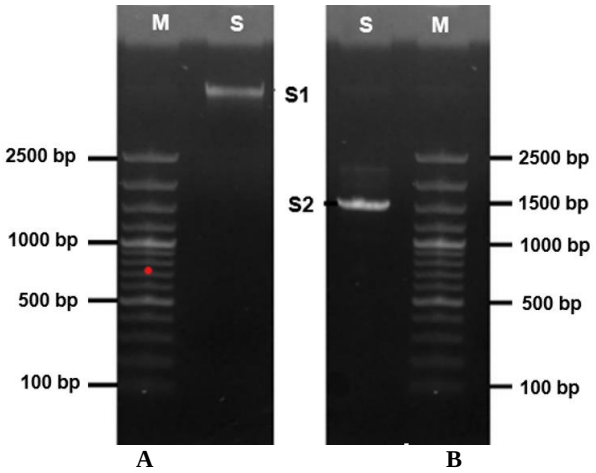

Figure 1. The electrophoresis results of DNA genome (A) and 16S rRNA gene (B) of Bacillus sp. BK7.1 on 1% agarose gel. Descriptions: M 100 bp DNA marker, S sample, S1 sample of DNA genome, S2 sample of 16SrRNA gene

Table 1. The species of Bacillus sp. BK7.1 based on approach 16S rRNA gene with Basic Local Alignment Search Tools (BLAST) program

Species	Accession no.	E value	%ID	Query cover (%)
Bacillus subtilis subsp. *inaquosorum strain* BGSC 3A28	NR_104873.1	0.0	98.68	99
Bacillus subtilis strain DSM 10	NR_027552.1	0.0	98.61	99
Bacillus subtilis strain JCM 1465	NR_113265.1	0.0	98.61	99

Table 2. Value of surface tension (mN/m) of supernatant fraction of *Bacillus subtilis* BK7.1 on treatment variation

Treatment	Surface Tension
Control of sterile water	72
Control of Nutrient Broth (NB) medium, room temperature, pH = 7	64.38
Control of Tween 20 at 50% solution	37.11
Supernatant of *Bacillus subtilis* BK7.1 (24 hours), room temperature, pH = 8	49.17
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against sterile water	22.83
Δ Surface tension of supernatant of *Bacillus subtilis* BK7.1 against NB medium	15.21

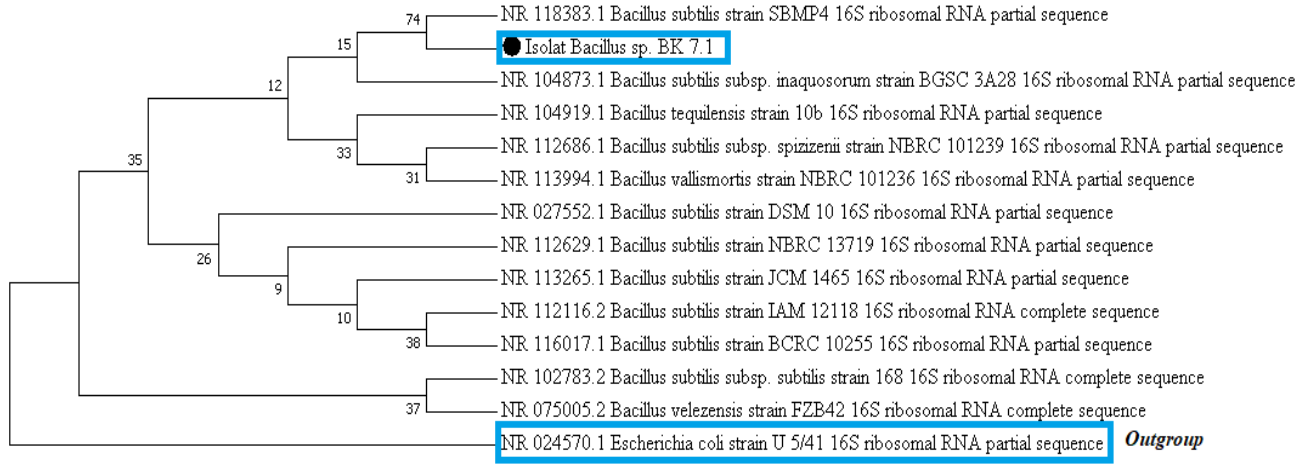

Figure 2. Analysis of the phylogenetic tree of *Bacillus* sp. BK7.1 based on cladograms of other species and strains, and *Escherichia coli* as an outgroup species

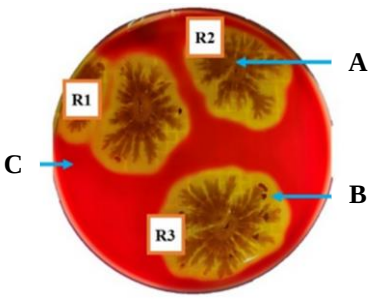

Figure 3. Screening biosurfactant using hemolytic activity in *Bacillus subtilis* BK7.1 on blood agar plate media. A. Isolate, B. Clear zone around the colony, C. Blood agar plate, R. Replicates

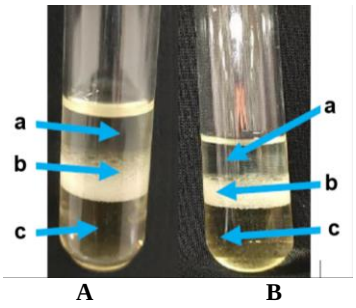

Figure 4. The emulsification activity of supernatant *Bacillus subtilis* BK7.1 on the kerosene substrate. A. 1 hour of exposure, B. 24 hours of exposure. a. kerosene, b. emulsion, c. isolate

Detection *srfAA* and *srfAD* surfactin gene

The encoding gene of surfactin discovered sizes scale 201 bp, expected as *srfAA* gene, and 723 bp expected as *srfAD* gene (Figure 5). In Table 3 showed that the similarity results, which have a value of 91.04% because there are several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. This can be caused by the presence of gene diversity even in the same *B. subtilis* group.

Biosurfactant production

Biosurfactant productions of *B. subtilis* BK7.1 on glucose, glycerol, and molasses substrates can be detected through a bacterial growth curve, surface tension value, and emulsification activity (Figure 6). The growth activity of *B. subtilis* BK7.1 showed on various substrates in Figure 6a. The isolates had grown well on SMW media with the addition of glucose, glycerol, and molasses as substrates. On glucose substrate with up to 72 hrs incubation, isolates still showed an exponential phase, and 96 hrs incubation entered the stationary phase, as well as on glycerol substrate. On molasses substrate, it still showed an exponential phase until 96 hrs incubation.

The results showed that the emulsification activity of *B. subtilis* BK7.1 on the three substrates tended to increase up to 96 hrs of incubation (Figures 6.B, 6.C), which proved that the isolate produced surfactin. On glucose substrate, the highest emulsification activity occurred at 96 hrs of incubation. Decreased in surface tension values are shown in Figure 6.D.

Table 3. The results of Basic Local Alignment Search Tools (BLAST) analysis of *srfAA* and *srfAD* protein isolates of *Bacillus subtilis* BK 7.1

Protein	Species	Accession no.	E value	%ID	Query cover (%)
Surfactin non-ribosomal peptide synthetase *srfAA*	*Bacillus subtilis inaquosorum*	WP_060397903.1	9e-34	91.04	100
Surfactin biosynthesis thioesterase *SrfAD*	*Bacillus subtilis* group	WP_075750164.1	5e-178	99.17	99

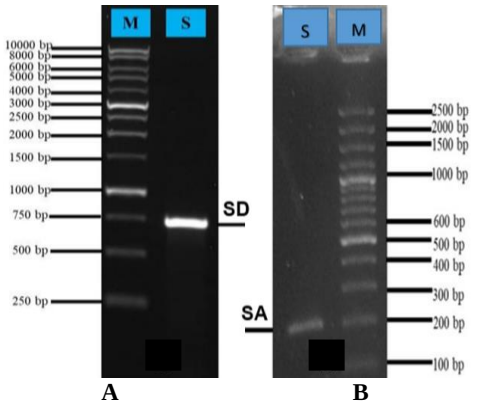

Figure 5. The electrophoresis results of *srfAD* (a) and *srfAA* (b) surfactin gene amplification of *Bacillus subtilis* BK7.1. Description: M 100 bp DNA marker, SA sample of *srfAA* surfactin gene 201 bp, SD sample of *srfAD* surfactin gene 729 bp

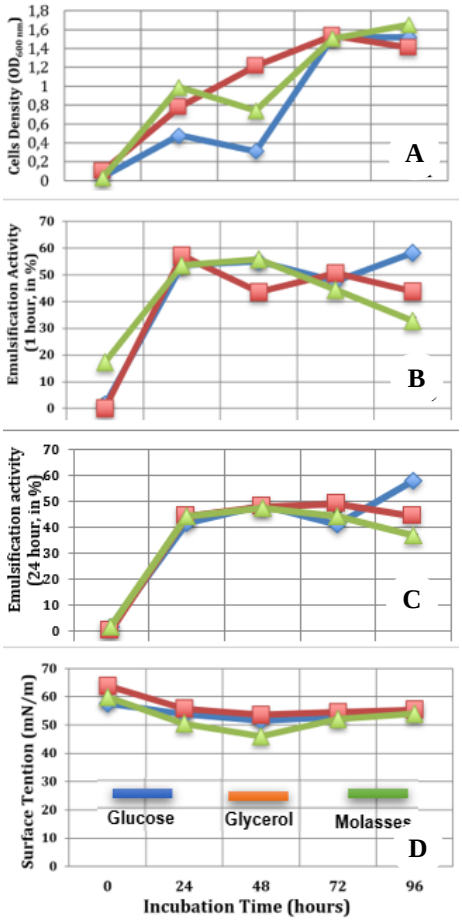

Figure 6. Biosurfactant productions of *Bacillus subtilis* BK7.1 on glucose, glycerol, and molasses substrates, incubation period 0-, 24-, 48-, 72-, 96-hours. Descriptions: A. Cells density, B. Emulsification activity (1 hour), C. Emulsification activity (24 hours), D. Surface tension value

Discussions

Conventional identification of *Bacillus* sp. BK7.1 has been carried out. Based on the macroscopic, microscopic, and physiological characteristics of *Bacillus* sp. BK7.1 has similarities with *Bacillus sphaericus* (Salamun et al. 2020). Researchers suggest further research to confirm the species name, by identifying the 16S rRNA gene. The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK7.1 showed a band over 1500 bp in size (Figure 1). *Bacillus* sp. BK7.1 had a 98.68% similarity to *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28 based on molecular identification. The gene of 16S rRNA could be used for the identification of microorganisms because it is one of the genes with specific characteristics (Pearson 2013). The 16S RNA gene sequencing is was a fast and accurate method for bacterial identification. Bacteria represented the same genus if they have a similarity index above 95% and the same species above 97% (Johnson et al. 2019; Srinivasan et al. 2015). The similarity was less than 100% because there were variations in amino acid sequences that affect the genotypic character but do not affect the phenotypic character (Johnson et al. 2019).

Research has shown that *B. subtilis* strain SBMP4 could control pathogenic fungi such as *Aspergillus* and *Fusarium* in early *Arachis hypogea* plants (Syed et al. 2020). *Bacillus* has adapted to and grown in extreme environmental conditions, forms endospores that are resistant to stress, and secretes various secondary metabolites such as surfactin (Shafi et al. 2017). Another essential characteristic was the abundance of secondary metabolites and moderate dietary requirements with a fast growth rate (Yadav et al. 2016; Mishra and Arora 2018). Biosurfactant lipopeptides from entomopathogenic microbes could act as biocontrol, especially antimicrobials and anti-biofilms (Abdel-Aziz et al. 2020; Qureshi et al. 2021). Surfactin produced by *B. subtilis* was one of the most effective biosurfactants. Surfactin reduced the surface tension of water up to 27 mN/m, with a critical micelle concentration of 0.01 g/L and high emulsification activity and has shown antimicrobial, antiviral, and antitumor activity (Gudina et al. 2013, 2015).

Controlling insects can use biosurfactants introduced as an alternative to synthetic chemicals. Many reports that biosurfactant activity produced by the *Bacillus* strain could kill adult mosquitoes. The hemolytic activity of *B. subtilis* BK7.1 could be seen in Figure 3. The clear zone on the hemolytic activity test by biosurfactants has caused lysis of the red blood cell membrane, and the cells secrete hemoglobin. The hemolytic activity occurred through two different mechanisms, at a high concentration occur, cell membrane lysis, and at low concentrations increase, membrane permeability to solutes and cause osmotic lysis

(Zaragosa et al. 2010). The inhibition zone formed in the observation of hemolytic activity indicates a biosurfactant production process; the larger the lysis diameter of blood agar, the higher the biosurfactant concentration (Singh 2012).

Bacteria could produce biosurfactants if they can reduce surface tension values by ≥10 mN/m (Oliveira et al. 2021). The surface and interfacial tension decrease is caused by the presence of hydrophobic and hydrophilic groups in the biosurfactants, where these compounds can accumulate between the liquid phases (Kapadia and Yagnik 2013). The entomopathogenic activity of biosurfactants against *A. aegypti* was caused by surfactin produced by *B. subtilis*. Surfactin triggers the surface tension of the water, causing a lack of oxygen underwater. The concentration of O_2 caused the larval spiracles of *A. aegypti* to open so that it can cause the insect death. In addition, surfactin could be very active against pH, temperature around 25-42°C, and UV stability, making it enjoyable to develop as a larvicidal agent (Guimarães et al. 2019).

The emulsification index value of *B. subtilis* BK7.1 was a low category. Lipopeptides such as surfactin consist of cycloheptapeptides with amino acids attached to fatty acids of a different chain. This chemical structure caused surfactin to be amphiphilic and able to mix in both polar and non-polar solvents, while this amphiphilic structure allows surfactin to form emulsions. The characteristics of surfactin were involved in cell attachment and cause membrane disruption (Chen et al. 2022). The ability of surfactin to bind Ca^{2+} caused a conformational change in the peptide cycle and allows it to be incorporated into the phospholipid bilayer (Khedher et al. 2015, Khedher et al. 2017).

The emulsification activity of *B. subtilis* BK7.1 in 1-hour observation tended to decrease compared to 24 hrs observation. This difference has shown that the emulsion was unstable because the isolate produces biosurfactants which act as active surface molecules only in decreasing surface tension. Based on molecular weight, biosurfactants have been classified into low and high molecular weight biosurfactants. Low molecular weight biosurfactants, including glycolipids, phospholipids, and lipopeptides, were efficient in reducing surface tension. Meanwhile, high molecular weight biosurfactants, such as proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers, were more effective in stabilizing oil-in-water emulsions as emulsifiers (Uzoigwe et al. 2015). This result was supported by the fact that the biosurfactant produced by *B. subtilis* 21332 has shown high emulsification activity values on glucose substrates up to 55.2% (Zhu et al. 2016). In contrast to the reported that value of the emulsification activity of *B. subtilis* 573 to 27.1%, with the addition of 1% bacterial culture concentration (Pereira et al. 2013), while in this study, the addition of 4%. Differences in the addition of culture affected the activity of biosurfactants produced by bacteria. The higher the concentration of bacterial culture added to the media, the density of bacteria in the substrate also increases and affects the speed of using the available substrate to produce biosurfactants.

Bacillus species had *srfAA* gene, which encodes phosphopantetheinyl transferase and contributes to the nonribosomal biosynthesis of surfactin (Plaza et al. 2015). The nonribosomal peptide synthetase complex was coded by *srfAA* and *srfAD* gene known as surfactin synthetase. The *srfAA* and *srfAD* genes have contributed to the control of surfactin biosynthesis gene expression. The 4-phosphopantetheinyl transferase was an activating enzyme for the srfA multienzyme complex. The *srfAA*, srfAB, srfAC, and *srfAD* genes were involved in the assembly of heptamodular non-ribosomal peptide (NPRS) synthesis in which the modular enzyme contains a typical N-terminal in the CLP-BGCs domain and acylates the first amino acid, glutamine with various 3-OH fatty acids derived from of primary metabolism (Théatre et al. 2021). The surfactin gene transformed surfactin synthetase into an active form. The production of biosurfactants especially surfactin, that have *Bacillus* influenced by *srfAA* and *srfAD* gene (Plaza et al. 2015). Table 3 showed that the similarity results have a value of 91.04%, because there were several differences in amino acids possessed by *B. subtilis* BK 7.1 and other strains of *B. subtilis*. The presence of gene diversity could cause this even in the same *B. subtilis* group.

The results of this study have also reported that there are differences in the production of biosurfactants. The higher emulsification activity from *B. subtilis* 573 to 48.4% (Pereira et al. 2013), *B. subtilis* 21332 up to 55.2% (Zhu et al. 2016), and *B. subtilis* N3-4P up to 38.3% (Zhu et al. 2016) on mineral salt media containing using different carbon sources than glycerol. The production of biosurfactant by *Bacillus nealsonii* S2M in glycerol substrate has been able to emulsify various hydrocarbons in 55% (Phulpoto et al. 2020).

Biosurfactant production of *B. subtilis* BK7.1 observed through surface tension values is shown in Fig. 6d. Glucose and sucrose substrates have been reported as the best carbon sources for the biosurfactant production process by the *Bacillus* group (Abdel-Mawgoud et al. 2008). *B. subtilis* BK7.1 reduced the surface tension up to 51.47 mN/m at 48 h incubation. *B. subtilis* B30, in 2% glucose substrate has the lowest surface tension value (25.56 mN/m) (Al-Wahaibi et al. 2014). The difference in surface tension reduction was caused by different species and strains of bacteria, as well as the level of their ability to utilize various substrates. Variations in nucleotide sequences between bacteria species affected the formation of biosurfactant biosynthetic genes.

On the glycerol substrate, *B. subtilis* BK7.1 has reduced the surface tension to 53.67 mN/m at 48 h, 42.01 at 96 h, and 54.36 at 72 h incubation, respectively. *B. subtilis* N3-4P has grown better on glycerol substrate than glucose, hexadecane, and diesel. This *B. subtilis* N3-4P decreased the surface tension to 27.8 mN/m on glycerol substrate (Zhu et al. 2016). The same has been reported that the difference in the value of the decrease in surface tension by *B. subtilis* 309, *B. subtilis* 311, and *B. subtilis* 573 on glycerol and glucose substrates, with the value of the decrease in surface tension on glycerol substrates 29.7, 30.1, and 29.9 mN/m, but on glucose substrates 29.2, 29.0, and 29.5 mN/m, respectively (Pereira et al. 2013).

The value of the surface tension of *B. subtilis* BK7.1, on molasses substrate, was 45.91 mN/m. *B. subtilis* SNW3 on molasses substrate was able to reduce the surface tension up to 41 mN/m (Umar et al. 2021), *B. subtilis* ATCC 6633 up to 30.48 mN/m (Kashkouli et al. 2011), and *B. subtilis* RSL-2 up to 24.09 mN/m (Verma et al. 2020). This difference has been due to the influence of various concentrations of molasses substrate, *B. subtilis* BK7.1 used 2% molasses, *B. subtilis* ATCC 6633 used 3% molasses (Kashkouli et al. 2011), and *B. subtilis* RSL-2 used 5% molasses (Verma et al. 2020). In addition, the efficiency of biosurfactant production by *B. subtilis* 3KP with molasses substrate was influenced by the instability of the biosurfactant product. Differences in composition and nutrient content in molasses, suspected related to the processing of sugar from the molasses (Ni'matuzahroh et al. 2017). The difference in sugar content of molasses as the main carbon source for the growth of *B. subtilis* 3KP bacteria has affected the productivity of biosurfactant production (Ni'matuzahroh et al. 2017).

Indigenous entomopathogenic *B. subtilis* BK7.1 isolated from Baluran National Park, East Java, Indonesia, 98.68% similarity to *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28. The results of screening for biosurfactant activity showed positive hemolytic activity, decreased surface tension, and increased emulsification activity. The *srfAA* and *srfAD* genes were detected encoding surfactin, which has the capacity for biosurfactant production on various glucose, glycerol, and molasses substrates. *B. subtilis* BK7.1 produced biosurfactant, the potential to develop for environmentally friendly biocontrol agent for biopesticides in agriculture and disease vector control in public health. Therefore, this research needs to be followed up to detect the chemical components of biosurfactants produced by these bacteria.

ACKNOWLEDGEMENTS

The authors would like to express appreciation for the financial support from the internal funding Featured Faculty Research (*Penelitian Unggulan Fakultas*) 2022-2023 and Featured Basic Research (*Penelitian Dasar Unggulan*) 2023 Universitas Airlangga. The authors grate full to the Dean of the Faculty of Science and Technology (*Fakultas Sains dan Teknologi*) and Head of Research and Community Services Institutions (*Lembaga Penelitian dan Pengabdian Masyarakat*), and Chancellor of Universitas Airlangga, Surabaya, Indonesia.

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